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13. ABSTRACT (Maximum 200 Words)

The epidermal growth factor receptor (EGFR) is an important mediator of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) which must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. The *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.* In year 2, we demonstrated that LAR expression is regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. Additionally, we showed that cell contact inhibits EGF-dependent proliferation in MCF10A mammary tumor cells and is mediated at the level of Akt activation. E-cadherin complexes are presumed to be the mediators of this cell contact inhibition. In year 3, we have shown that cell contact inhibition of Akt blocks the cell cycle. The central role of Akt regulation in this pathway is confirmed by recapitulating the effect with an adenovirus-mediated expression of a dominant negative Akt. Finally, transgenic expression of LAR in mammary epithelium under the MMTV promoter did not generate abnormal rates of spontaneous tumors. Taken together, these data indicate that the critical regulation of EGF signaling is not at the receptor via LAR but downstream at Akt.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) and the related protein erbB2 have been implicated as important mediators of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) that must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. Two sets of data are particularly important. First, the LAR knockout mouse has been shown to have a defect in terminal mammary gland development. Second, we have shown that suppression of cellular LAR by 60% using an antisense expression vector results in a 3-4 fold elevation of EGF-dependent receptor signaling. Based upon these and other observations, the *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.*

ANNUAL REPORT

The research question (hypothesis) presented in this proposal is that *LAR plays an important role in EGFR-dependent breast cancer through negative modulation of EGFR signal transduction*. With the long term goal of understanding the mechanisms by which EGF signaling is abnormal in breast cancer, two specific aims have been pursued: **First**: characterize the impact of LAR on TGFα-dependent abnormal mammary gland development. This requires a mouse model which has the combined characteristics of a LAR deficient (knockout) mouse and a mouse which has a strong tendency to abnormal mammary development and tumors because of increased expression of the tumor-promoting gene (TGFα) in the mammary gland. The hypothesis would predict that abnormal mammary development will be more pronounced in those mice with the absence of the modulatory effects of LAR. **Second**: define the mechanism by which LAR mediates its modulatory effect on EGFR signaling and alters mammary gland development. Examine how LAR interacts with EGFR. Is it direct or through some intermediate steps?

Aim #1: Characterize the influence of PTP LAR on TGFα-dependent abnormal mammary gland development.

This objective required the crossing of LAR knockout mouse with mice expressing TGFα targeted to the mammary epithelium. These studies required the crossbreeding of the mice and monitoring them for tumor formation for a period of 12 months and beyond. Co-investigator, Dr. William Kisseberth, who was to accomplish this aspect of the project, relocated to the Dept. of Clinical Sciences, College of Veterinary Medicine, Ohio State University. It was initially decided to initiate these studies once Dr. Kisseberth re-established at Ohio State. With the unexpected delay due to Dr. Kisseberth's relocation, it became clear that this time extensive Specific Aim could not be accomplished in the remaining grant funding period. A more practical objective was designed. This goal was to thoroughly investigate a transgenic mouse model that was established during Year 1. In this mouse model, a human LAR transgene is targeted specifically to mammary epithelium. The experimental question to be asked is: Does LAR regulate pregnancy-dependent mammary gland maturation? This is very relevant to the hypothesis of this project since EGF receptor family members are critical to mammary gland maturation. Additionally, it has been reported that LAR knockout mice have impaired mammary gland development. The added benefit of this revised investigation is that the endpoint is mammary gland development during pregnancy, not a year of monitoring for tumor development.

The steps followed to develop this transgenic mouse are a follows:

- Obtained MMTV cassette in a pSP73 vector from Dr. Kai-Shun Chen at the University of Wisconsin
- Obtained the human LAR gene in the pSP6 vector from Dr. Michel Streuli of the Dana Farber Cancer Institute
- Subcloned the human LAR cDNA minus the polyA sequence (a 6.5 kb fragment) into the MMTV cassette
- Excised and purified the MMTV-LAR cassette (11.2 kb) from the pSP73 vector
- The linear MMTV-LAR construct was injected into mouse blastocysts and placed in a recipient mice.

Six founder mice have been obtained as determined by detection of SV40 poly A sequence by PCR. 5 of 6 founder mice had germ-line integration of the transgene. 1 of 6 was mosaic; however, germ-line transmission was established from F1 offspring. Human LAR message was detected in the mammary glands of all founder lines using a RT-PCR assay that discriminates between mouse and human LAR message (figure 1).

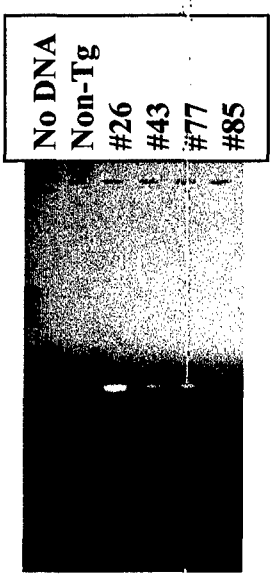

Fig. 1. Mammary gland RT-PCR Expression of MMTV-hLAR. RT-PCR analysis of human LAR expression using human-specific oligonucleotide primers. Total RNA was isolated from day 18 pregnant MMTV-LAR transgenic mice from four different transgenic lineages.

Targeted expression of LAR to mammary epithelium, in preliminary investigations, does not have overt effects on virgin or pregnancy-dependent mammary development (figure 2). MMTV-LAR transgenic mice do not appear to have an increased incidence of mammary neoplasia.

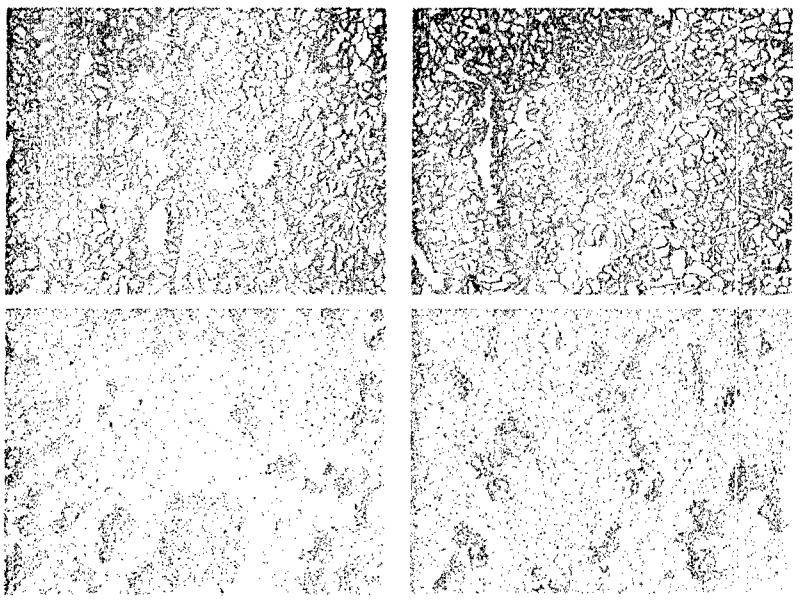

Figure 2. Mammary gland development in MMTV-LAR transgenic mice. The histomorphological appearance of MMTV-LAR transgenic mammary glands are identical to nontransgenic mice. *A,* day 18 pregnant non-transgenic: *B,* day 18 pregnant MMTV-LAR (line

101-26); *C,* day 4 post-lactation, non-transgenic; *D,* day 4 post-lactation MMTV-LAR (line 101-26).

Ongoing studies to further characterize these mice include:

- Create MMTV-LAR/WAP-TGFα and MMTV-LAR/MMTV-c-neu bitransgenic mice and determine if over-expression of LAR alters the mammary gland phenotypes observed in single transgenic WAP-TGFα and MMTV-c-neu mice.
- Determine if LAR over-expression alters tumorigenesis in bitransgenic mice.

These studies should help to indirectly support the hypothesis that increased LAR expression may act as a tumor suppressor in neu expressing mammary epithelia and tumors as suggested by Zhai (1993).

Aim 2: Elucidate the mechanism by which LAR modulates EGFR signaling in the mammary gland.

Two aspects of Aim #2 have shown accomplished during Year #3.

A) our investigation into the regulation of LAR expression by cell density and E-cadherin complexes has been published:

> Symons, J., LeVea, C., and Mooney, R. (2002) Expression of the leucocyte common antigen-related (LAR) tyrosine phosphatase is regulated by cell density through functional E-cadherin complexes. Biochem. J. 365,513-519.

B) an investigation of the mechanism by which cell contact inhibition (mediated by functional E-cadherin complexes) inhibits EGFR proliferation signaling in a mammary epithelial cell model has been completed and the manuscript has been submitted:

> LeVea,C. and Mooney, R. (2003) EGF-dependent cell cycle progression is controlled by density-dependent regulation of Akt activation. Submitted to *Exper. Cell Research*

A) Expression of PTP LAR is Regulated by Cell Density Through Functional E-Cadherin Complexes: We have completed this investigation. An Abstract is included below. A reprint is included in the Appendices.

The receptor-like protein tyrosine phosphatase LAR has been implicated in receptor tyrosine kinase signaling pathways while also displaying cell density dependency and localization to adherens junctions. While physiologic substrates for LAR have not been unequivocally identified, β-catenin associates with LAR and is an *in vitro* substrate. With the implication that LAR may play a role in regulating E-cadherin dependent cell-cell communication and contact inhibition, the relationship of LAR to E-cadherin was investigated.

LAR expression increased with cell density in the human breast cancer cell MCF-7 and in Ln 3 cells derived from the 13672NF rat mammary adenocarcinoma. LAR protein levels rapidly decreased when cells were replated at low density after attaining high expression of LAR at high cell density. COS-7 cells displayed comparable density-dependent regulation of LAR expression when transiently expressing exogenous LAR under the control of a constitutively active promoter, indicating that the regulation of expression is not at the level of

gene regulation. Disrupting homophilic E-cadherin complexes by chelating extracellular calcium caused a marked decrease in LAR protein levels. Similarly, blocking E-cadherin interactions with saturating amounts of E-cadherin antibody (HECD-1) also led to a rapid and pronounced loss of cellular LAR. In contrast, mimicking cell surface E-cadherin engagement by plating cells at low density onto dishes coated with HECD-1 resulted in a 2 fold increase in LAR expression compared to controls. These results suggest that density-dependent regulation of LAR expression is mediated by functional E-cadherin and may play a role in density-dependent contact inhibition by regulating tyrosine phosphorylation in E-cadherin complexes.

B) EGFR signaling is inhibited by cell contact at the level of Akt activation: This investigation is now complete. A key series of experiments which employed an adenovirus construct expressing a dominant negative Akt, was used to convincingly demonstrate that Akt activity is essential for EGF–dependent cell cycle progression. This strengthened the conclusions of the investigation. These data are as follows:

Akt activation is necessary for cell cycle progression: Low-density cells, which had been infected with an adenovirus expressing both dominant-negative Akt and green fluorescent protein [16] or with an adenovirus containing only control genes, were treated +/- EGF for 21 hr. Subsequently, the cells were separated by fluorescence activated cell sorting to isolate the dominant-negative Akt infected cells (expressing green fluorescent proteins). The dominant-negative Akt infected cells, control adenovirus infected cells, and uninfected cells were subjected to cell cycle analysis. As can be seen in fig 11, EGF stimulated the proliferative fraction (S and G2/M) in the uninfected cells from 19% to 44% and in the adenovirus vector control infected cells from 28% to 45%. However, the dominant-negative Akt expressing cells were blocked from EGF-dependent cell cycle progression. They showed a proliferative fraction that only increased from 16% to 27%. The comparison among the three conditions demonstrates that EGF-dependent Akt activation is necessary for cell cycle progression.

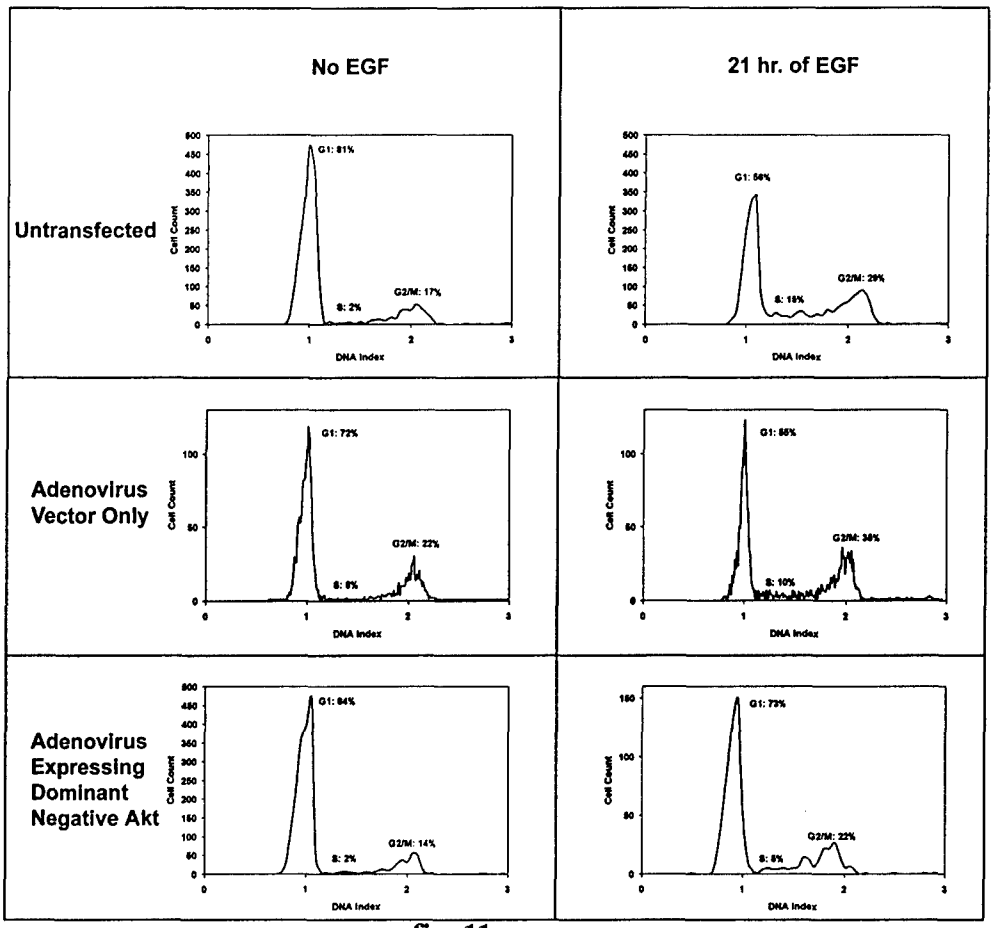

fig. 11

Fig. 11. Adenovirus mediated expression of dominant-negative Akt prevents cell cycle progression in low-density cells. Low-density cells were infected with adenovirus containing dominant-negative Akt or control adenoviral DNA. These infected cells plus uninfected control cells were treated +/- 5 ng/ml EGF for 21 hr and processed for cell cycle analysis as described in the *Materials and Methods*. DNA histograms were obtained from slides analyzed on an Oncometrics Cyto-Savant automated image cytometer. DNA content was measured according to the Oncometrics protocol using thionine as the DNA stain. The calculated sum optical density (DNA index) was plotted vs. frequency (cell count).

Abstract: The normal human breast epithelial cell line, MCF10A, was used to investigate the mechanism by which high-density intercellular contacts inhibit EGF-dependent cell cycle progression. EGF-dependent Akt activation was found to be transient in high-density cells and sustained in low-density cells. High-density cells also showed decreased EGF receptor (EGFR) autophosphorylation, decreased retinoblastoma protein phosphorylation, and increased p27 protein expression. Although EGFR activation was decreased in the high-density cells, the activation was sufficient to stimulate EGFR substrates comparable to low-density cells. EGF-dependent activation of the Erk1/2 pathway and the upstream activators of Akt (Gab1, erbB3, PI3 kinase, and PDK1) showed no density dependency. Antagonists of Akt activity provided further evidence that regulation of Akt activation is the critical signal transduction step controlling EGF-dependent cell cycle progression. Both adenovirus-mediated

expression of dominant-negative Akt and inhibition of PI3 kinase-mediated Akt activation with LY294002 blocked cell cycle progression of low-density cells. In summary, intercellular contacts appear to block EGF-dependent cell cycle progression by inhibiting EGF signaling at the level of EGF-dependent Akt activation.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that expression of the LAR phosphatase in the human breast cancer cell line, MCF7, is dependent upon cell-cell contact.
- Characterized the mechanism by which LAR protein is regulated and demonstrated that functional E-cadherin complexes are necessary and sufficient for this effect.
- This is particularly relevant to the objectives of this project because engagement of E-cadherin complexes are also known to exert an inhibitory effect on EGF receptor signaling, particularly mitogenesis. Might it be that the E-cadherin effect is, in part, mediated through the expression of the PTP LAR as our hypothesis would suggest?
- Cell contact inhibition of EGF-dependent growth is mediated at the level of Akt activation.
- Akt activation if a critical step for control of cell cycle progression, and this inhibition by contact inhibition is present for at least 21 hr after exposure to EGF.
- Targeted expression of LAR to mammary epithelium, in preliminary investigations, does not have overt effects on pregnancy-dependent mammary development.

REPORTABLE OUTCOMES

Publication:
Symons, JR, LeVea, C, and Mooney, RA (2002) Expression of PTP LAR is regulated by cell density through functional E-cadherin complexes. *Biochem. J.* 365, 513-519.

Manuscript submitted:
LeVea, C, and Mooney, RA (2002) Cell contact inhibition of growth is mediated by E-cadherin dependent suppression of EGF-stimulated Akt signaling.

Mouse transgenic model:
Targeted expression of human LAR selectively to the mammary gland via the MMTV promoter.

CONCLUSIONS

Work in the third year has completed mechanistic investigations that have addressed the hypothesis that LAR is a negative regulator of EGF receptor signaling. We have characterized the regulation of cellular LAR expression and have revealed an important role for E-cadherin in this process. LAR is regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. Tyrosine phosphorylated proteins in the E-cadherin complex may be substrates for LAR. Thus, LAR may regulate E-cadherin complex function. How does this regulate EGF receptor signaling? It is well known that the proliferative effect of EGF receptor signaling is suppressed at confluence, presumably by E-cadherin complexes. We now have shown that EGF proliferative signaling is critically inhibited at the level of Akt activation. Somewhat surprisingly, regulation of EGF receptor tyrosine phosphorylation and substrate phosphorylation appears less critical. An unanswered questions is by what mechanism do cell surface interactions involving E-cadherin, and presumably LAR, mediate their effects directly on Akt activity state.

Characterization of mammary gland development as a function of LAR expresssion is continuing during a grant extension. These studies involve transgenic mice with targeted expression of human LAR to the mammary epithelium under the control of the MMTV promoter. These studies will yield important information in the coming months in the laboratory of Dr. Kisseberth at Ohio State.

References: NA

Appendices: 1 reprint

1 manuscript

Expression of the leucocyte common antigen-related (LAR) tyrosine phosphatase is regulated by cell density through functional E-cadherin complexes

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The leucocyte common antigen-related phosphatase (LAR) has been implicated in receptor tyrosine kinase signalling pathways while also displaying cell-density-dependency and localization to adherens junctions. Whereas physiological substrates for LAR have not been identified unequivocally, β-catenin associates with LAR and is a substrate *in vitro*. With the implication that LAR may play a role in regulating E-cadherin-dependent cell–cell communication and contact inhibition, the relationship of LAR with E-cadherin was investigated. LAR expression increased with cell density in the human breast cancer cell line MCF-7 and in Ln 3 cells derived from the 13762NF rat mammary adenocarcinoma. LAR protein levels decreased rapidly when cells were replated at a low density after attaining high expression of LAR at high cell density. COS-7 cells displayed comparable density-dependent regulation of LAR expression when transiently expressing exogenous LAR under the control of a constitutively active promoter, indicating that the regulation of expression is not at the level of gene regulation. Disrupting homophilic E-cadherin complexes by chelating extracellular calcium caused a marked decrease in LAR protein levels. Similarly, blocking E-cadherin interactions with saturating amounts of E-cadherin antibody (HECD-1) also led to a rapid and pronounced loss of cellular LAR. In contrast, mimicking cell-surface E-cadherin engagement by plating cells at low density on to dishes coated with HECD-1 resulted in a 2-fold increase in LAR expression compared with controls. These results suggest that density-dependent regulation of LAR expression is mediated by functional E-cadherin and may play a role in density-dependent contact inhibition by regulating tyrosine phosphorylation in E-cadherin complexes.

Key words: β-catenin, MCF-7.

INTRODUCTION

E-cadherin is a member of a family of calcium-dependent transmembrane proteins which play a central role in cell–cell communication and contact inhibition [1,2]. These functions are mediated by homophilic binding between extracellular domains of E-cadherin expressed on neighbouring cells and localized to adherens junctions. The intracellular domain of E-cadherin contains binding sites for several proteins, including β-catenin, γ-catenin (plakoglobin) and p120 catenin. Tight cell–cell adhesion is achieved through these protein–protein interactions, which form a bridge to the actin filaments. The E-cadherin intracellular domain binds β-catenin, which in turn binds α-catenin. It is thought that α-catenin interacts directly or indirectly (via α-actinin) with the F-actin bundles, linking the complex to the actin cytoskeleton and forming a stable structure when laterally clustered [3].

Loss of E-cadherin in transformed epithelial cells has been correlated with increased tumour invasiveness *in vitro* and with tumour progression *in vivo* [4,5]. In breast cancer, loss of functional E-cadherin complexes correlates with a loss of the epithelial phenotype and increased invasiveness [6]. E-cadherin and the proteins within the cadherin complex are thought to be post-translationally modified to provide a mechanism for rapid turnover and modulation of the functional state. Several of these proteins, including p120 catenin, E-cadherin, γ-catenin (plakoglobin) and β-catenin, are apparently regulated by tyrosine phosphorylation [7–9]. Whereas tyrosine phosphorylation of β-catenin appears not to promote its uncoupling from cell adhesion complexes directly [10], it may be involved in a mechanism for disruption of functional E-cadherin complexes. Studies *in vitro* have identified several protein tyrosine phosphatases (PTPs) that bind to and alter the phosphorylation state of β-catenin. Among the kinases, the epidermal growth factor (EGF) receptor and erb-B2 associate with β-catenin *in vitro* and *in vivo* [11,12]. A β-catenin mutant lacking its N-terminal half, including much of the central armadillo motifs, cannot bind E-cadherin but binds to erb-B2. This mutant inhibits the tyrosine phosphorylation of endogenous β-catenin associated with the E-cadherin complex [12]. Cells transfected with this deletion mutant display decreased transforming growth factor-α-dependent migration *in vitro* and decreased metastasis *in vivo*. The hepatocyte growth factor (HGF) receptor/c-Met receptor has been shown to co-localize and associate with E-cadherin and β-catenin in epithelial tumour cells [13]. Following c-Met activation with HGF/scatter factor, tyrosine phosphorylation of β-catenin occurs and cell–cell contacts are disrupted [14]. The Src kinase has been implicated in this HGF-dependent process [15]. Rat fibroblasts become metastatic and lose their compact aggregating morphology when transformed with v-Src [16]. Tyrosine kinase inhibitors restore tight cell–cell adhesion. β-Catenin is the most significantly increased tyrosine-phosphorylated protein of the cadherin-associated proteins in the presence of v-Src [16]. Despite these observations, tyrosine phosphorylation of β-catenin as a mechanism for disruption of functional E-cadherin complexes remains controversial [10].

Several PTPs have also been shown to associate with cadherin complexes [17–21]. PTP activity appears to be necessary for strong cell–cell adhesion since cell–cell contact can be disrupted by treatment with the PTP inhibitor pervanadate [22]. Studies *in*

Abbreviations used: EGF, epidermal growth factor; HGF, hepatocyte growth factor; LAR, leucocyte common antigen-related phosphatase; PTP, protein tyrosine phosphatase; E-subunit, extracellular subunit; P-subunit, PTP-domain-containing subunit.

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vitro have demonstrated that leucocyte common antigen-related phosphatase (LAR) associates with the N-terminus of β-catenin and the closely related γ-catenin [17]. β-Catenin has also been shown to be a substrate for LAR *in vitro* [23]. These data suggest that LAR may play a role in the maintenance of cell–cell contacts by regulating the tyrosine-phosphorylation state of one or more proteins in the E-cadherin complex. Here, we expand upon the earlier report that LAR is regulated by cell density [24]. Functional E-cadherin complexes are shown to regulate expression of LAR through a mechanism that does not require changes in rates of transcription. Regulation of LAR expression may be a mechanism by which E-cadherin complexes control their own functional state.

EXPERIMENTAL

Cell lines

COS-7 green monkey kidney cells were obtained from the A.T.C.C. Ln 3 rat mammary adenocarcinoma cells were a generous gift from Dr Carl McGary (University of Rochester, Rochester, NY, U.S.A.). MCF-7 cells were provided by Dr P. J. Simpson-Haidaris (University of Rochester).

Plasmids and transfections

The mouse E-cadherin construct was a generous gift from Masatoshi Takeichi of Kyoto University, Kyoto, Japan, and has been described previously [25]. Human LAR cDNA (kindly provided by Michel Streuli of the Dana Farber Cancer Institute, Boston, MA, U.S.A.) was cloned into the pcDNA3 vector in-frame with a 5′ Myc/His tag. Bacterial transformation was performed using subcloning efficiency DH5α from Life Technologies. Plasmid preparations were performed using Qiagen maxi-prep kits according to their protocol. DNA concentration and purity were measured by UV spectroscopy. Transient transfections of cell lines were performed using the Fugene 6 reagent (Roche) under serum-free conditions as described by the manufacturer. Transfected cells were maintained for 36 h before experimentation.

Antibodies and reagents

Mouse monoclonal antibodies specific for β-catenin, E-cadherin and the LAR E-subunit were from Transduction Laboratories. The anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Antibody for the LAR P-subunit (PTP-domain-containing subunit) was provided by Dr Barry Goldstein of Thomas Jefferson University (Philadelphia, PA, U.S.A.). Antibodies to human E-cadherin (HECD-1) and rat E-cadherin (DECMA-1) were from Zymed. All other reagents were purchased from Sigma unless otherwise indicated.

Immunoprecipitations and Western blotting

When direct analysis of cell lysates was appropriate, cells were scraped into Laemmli's lysis buffer (156 mM Tris/HCl, 5% SDS and 2.5% glycerol) at 100 °C. After normalizing samples based on protein using the Markwell–Lowry protein assay, proteins were separated by SDS/PAGE.

When immunoprecipitation of proteins was required, cells were scraped into lysis buffer [1 M Tris/HCl, pH 7.4, 2 M NaCl, 1 M NaF, 10% Triton X-100, 200 mM PMSF, benzamidine, tetrasodium pyrophosphate, *p*-nitrophenylphosphate, pervanadate and a protease-inhibitor cocktail (Calbiochem)]. Lysates were passed 10 times through an 18-gauge needle. Samples were protein-normalized using the Bradford assay. The immunoprecipitating antibody was pre-bound to Protein G–Sepharose beads (Amersham Bioscience) before lysates were added at 4 °C for 1–4 h. Beads were washed three times with Triton wash buffer (1% Triton X-100, 100 mM Tris/HCl and 150 mM NaCl) before immunoprecipitates were eluted with boiling Laemmli sample buffer (78 mM Tris/HCl, 2.5% SDS, 2.5% glycerol and 2 mM dithiothreitol). Samples were transferred to either PVDF or nitrocellulose membranes and analysed by Western blotting. The blots were developed with either Amersham Bioscience or Pierce chemiluminescence reagents.

Cell-density-dependent plating assays

Following propagation to confluence, cells were rinsed, dispersed with 0.25% trypsin for 1–5 min and resuspended in standard serum-containing media. Cells were pooled and concentrated by centrifugation. Aliquots were re-plated at high (> 85%) or low (< 30%) density in 100 mm diam. dishes. At appropriate time points, cells were lysed and protein expression analysed by Western blotting as described above.

Disruption of E-cadherin complexes

MCF-7 cells were grown to confluence in 24-well dishes. Cell–cell contact was disrupted with either EGTA or HECD-1, a mouse monoclonal anti-E-cadherin antibody. For EGTA treatment, EGTA was added at 5 mM with a media change followed by incubations for 6 h or less. Incubations of 18 h and longer were found to be cytotoxic. To block E-cadherin more specifically, MCF-7 cells were incubated in media containing the E-cadherin-blocking antibody, HECD-1, at 1, 5 or 10 μg/ml for 18–24 h. Cells were examined by light microscopy at several time points to ensure cell viability and to record morphological changes induced by treatment. Protein expression was analysed as described above.

RESULTS

LAR protein levels change with cell density

Our previous studies indicated that LAR protein expression is cell-density-dependent [24]. In light of the observed association of LAR with constituents of the adherens junctions [17,23,26] and the defect in terminal differentiation of the mammary gland in LAR-knockout mice [27], the current study was initiated to explore the mechanism of cell-density-dependency of LAR using breast cancer cell lines. LAR protein levels were examined in the human MCF-7 breast carcinoma cell line and in a rat mammary adenocarcinoma cell line, Ln 3. Each cell type was grown for several passages at either high (85–100% confluence) or low (10–30% confluence) cell density. The MCF-7 cells were additionally examined at 40–60% confluence (medium density). Cell lysates were normalized for protein and separated on SDS/PAGE gels. In the human MCF-7 cell line, LAR protein increased markedly from low to high density as detected by Western blotting using an antibody to the LAR E-subunit (Figure 1A). It was possible that the difference in LAR E-subunit expression did not reflect expression of the catalytically active P-subunit. For example, the E-subunit alone may have been lost at low cell density due to proteolytic cleavage or shedding of this extracellular domain [26]. However, when MCF-7 lysates were probed with an antibody to the catalytic or P-subunit of LAR, comparable density-dependent changes in LAR expression were observed. Similarly, the highly metastatic Ln 3 cells, derived from the 13762NF rat mammary adenocarcinoma [28], exhibited a large increase in LAR protein from low to high density when detected with the P-subunit antibody (Figure 1B). The less

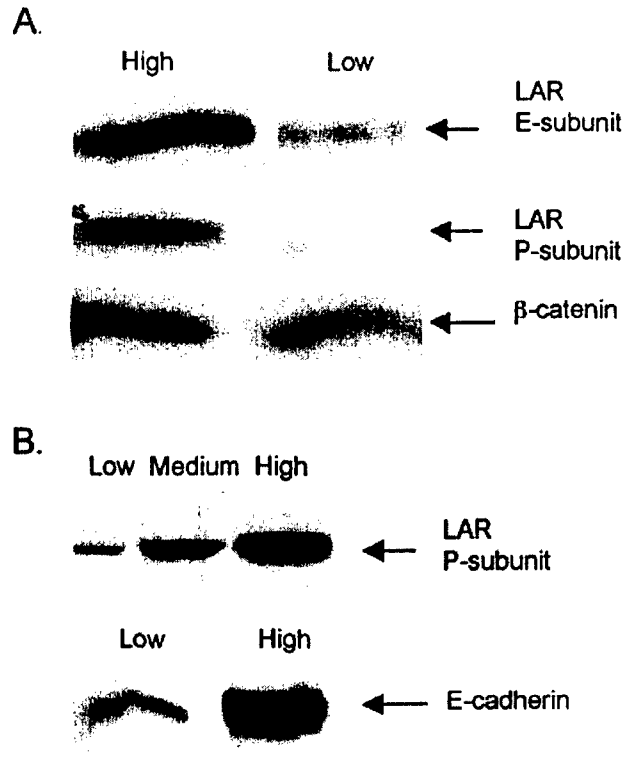

Figure 1 Effect of cell density on LAR and E-cadherin expression

MCF-7 (**A**) and Ln 3 (**B**) cell lines were grown for several passages at high (> 85%), medium (approx. 40–60%), or low (< 30%) confluence. After cell lysis, proteins were separated by SDS/PAGE and transferred to nitrocellulose. LAR subunit expression in MCF-7 cells (**A**) and expression of LAR and E-cadherin in Ln 3 cells (**B**) were detected by Western blotting as described in the Experimental section. β-Catenin was used as a loading control in (**A**).

metastatic Ln 2 cells, also derived from the 13762NF tumour, showed a progressive increase in LAR levels from low to high density similar to the Ln 3 cell line (results not shown). Analysis of E-cadherin levels as a function of cell density paralleled LAR levels in Ln 3 cells.

LAR levels respond to rapid changes in cell density

The following investigation examined the impact of acute changes in cell density on LAR expression. Cells were grown to confluence before being dissociated, pooled and replated at either high or low density. Within 4 h of replating, LAR protein levels decreased markedly when replated at low density. In contrast, replating at high density preserved the pre-existing LAR expression (Figure 2). E-cadherin levels decreased in parallel with those of LAR when cells were exposed to low-cell-density conditions. In contrast, β-catenin, which was used as a loading control, showed no density-dependent changes, though a small increase was observable as a function of time after replating. The decreased expression of LAR reflected changes in both the E- and P-subunits. Whereas the E-subunit potentially could have been influenced by shedding, the similar density-dependent changes in LAR observed in Ln 3 cells (Figure 2B) probed with either a P-subunit or E-subunit antibody argue that decreased cell density initiated the loss of total cellular LAR protein.

The above investigation demonstrated that LAR levels decreased markedly within 4 h of an acute decrease in cell density.

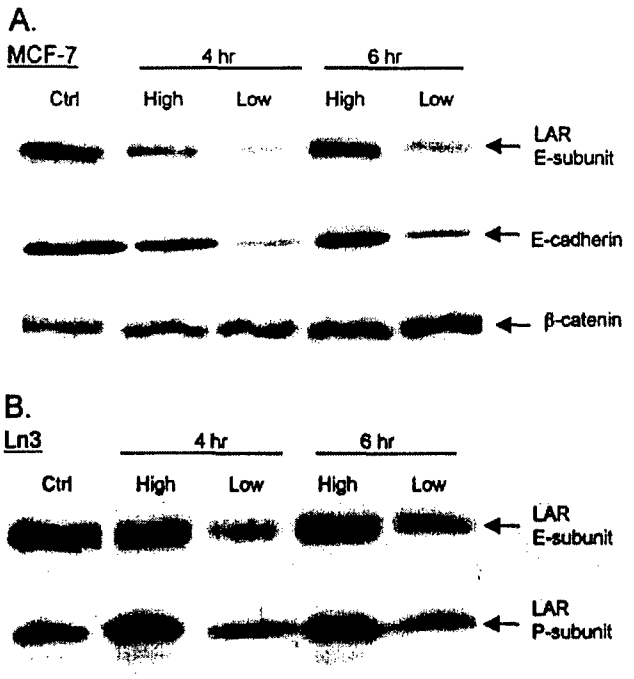

Figure 2 Rapid decrease in LAR expression with a decrease in cell density

MCF-7 (**A**) and Ln 3 (**B**) cells, which had been maintained at confluence, were resuspended with trypsin and replated at either high (> 85%) or low (< 30%) confluence for either 4 or 6 h. Changes in expression of LAR E-subunit (**A**, **B**), LAR P-subunit (**B**), E-cadherin (**A**) and β-catenin (**A**) were determined by Western blotting. Ctrl, control.

Next the response to an acute increase in cell density was investigated. Cells were maintained at 10–30% confluence for several days before harvesting and replating at high density. LAR protein expression was analysed by Western blotting at subsequent times as indicated in Figure 3. LAR was modestly increased within 8 h of replating at high density and continued to increase more substantially from 8 to 24 h. By 24 h, the level of LAR protein reached that of the high-density control cells (results not shown). Because MCF-7 cells do not proliferate rapidly and the cells had been plated at high density, the increase in LAR protein expression was not likely to be due to cell

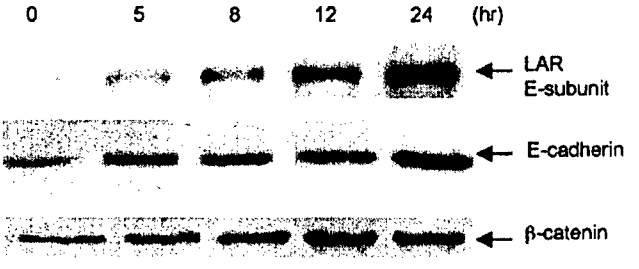

Figure 3 Slow increase in LAR expression with a rapid increase in cell density

MCF-7 cells were maintained at low density (< 30%) for several passages. Following resuspension with trypsin, pooled cells were replated at high cell density. After replating, LAR, E-cadherin and β-catenin expression were determined by Western blotting at the indicated time points.

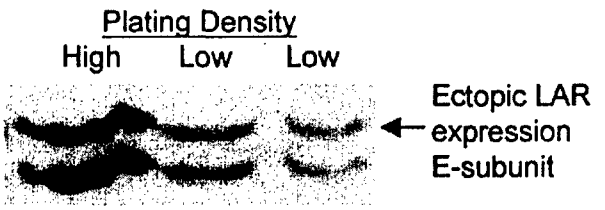

Figure 4 Density-dependent regulation of ectopically expressed LAR

COS-7 cells were transiently transfected with a human LAR cDNA construct as described in the Experimental section. At 36 h post-transfection, cells were resuspended with trypsin, pooled and replated at either high (> 85%) or low (< 30%) confluence for 6 h. Experiments with the low-confluence condition were performed in duplicate. LAR expression was determined by Western blotting.

proliferation. The more modest increase in β-catenin and the absence of an increase over the time period (12–24 h) that corresponds to the large increases in LAR and E-cadherin supports this conclusion. Interestingly, the most substantial increases in LAR protein expression occurred subsequent to the formation of cell–cell contacts. As observed by light microscopy, the MCF-7 cells began to spread at 4–6 h and visible cell–cell contacts were observable at 8–10 h. Whereas LAR levels increased substantially after 8 h, maximum levels were not reached for an additional 16 h. Thus the slow rate of increase in LAR mass with an acute increase in cell density is in marked contrast to the very rapid loss of LAR with acute decrease in cell density. It is logical to speculate that *de novo* synthesis of LAR, and perhaps other proteins, is required to mediate the observed changes. Finally, increases in LAR protein levels paralleled those of E-cadherin in response to the increase in cell density. From the above data, we hypothesize that LAR protein levels increase with the formation of functional cell–cell contacts.

Density-dependent changes in LAR levels do not require changes in the synthetic rate

To address the possibility that the difference in LAR levels at varying densities was due to differing rates of transcription, we used a method similar to that of Gebbink et al. [29]. COS-7 cells were transiently transfected with LAR cDNA under the control of a constitutively active promoter. The transfected cells were incubated for 24 h, dissociated and replated at either high (> 85%) or low (< 30%) density. The cells were lysed 24 h later and LAR expression was analysed. Since exogenous LAR expression is under the control of a constitutively active promoter, the transcription rate of LAR remains constant whether cells are at high or low densities. Changes in LAR levels would be due to altered rates of protein degradation or mRNA stability and translation. As shown in Figure 4, LAR expression in transfected COS cells, which express no detectable endogenous LAR, increased severalfold with increasing cell density. Thus regulation of LAR transcription rate is not responsible for density-dependent LAR expression. Ectopically expressed LAR in COS cells appeared as a doublet by Western blotting. One potential explanation is that these bands represent alternatively spliced isoforms of the extracellular domain of LAR. Zhang et al. [30] have described a 27 bp exon encoding a nine amino acid sequence in the E-subunit of LAR that is alternatively spliced. This group has shown that expression of these isoforms is expressed in a developmentally regulated pattern in the brain. While the co-expression of two LAR isoforms is not observed routinely in transfection experiments, doublets of the E-subunit have been observed by Western blotting following transfection [31,32]. An

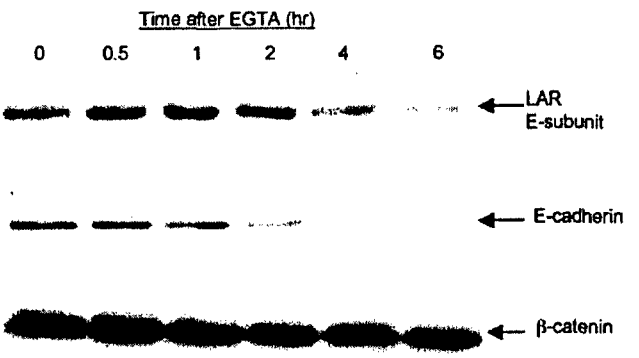

Figure 5 Effect of EGTA-dependent disruption of cell–cell contacts on expression of LAR and E-cadherin

Confluent MCF-7 cells were treated with 5 mM EGTA for the time periods indicated and then harvested. LAR, E-cadherin and β-catenin (loading control) expression were determined in the cell lysates using Western blotting.

alternative explanation for the presence of a doublet in LAR Western blots is that one or both represent *in vitro* proteolytic fragments. The ratio of the two immunoreactive bands remained relatively constant at approx. 1.0 among samples and between experiments. This and the absence of a doublet with endogenous LAR argue for a cell-dependent processing rather than an *in vitro* artifact, such as proteolysis.

LAR protein levels are altered by disruption of E-cadherin-mediated cell–cell contact

We hypothesized that cell-density-dependent increases in LAR protein expression were due to a signal mediated by E-cadherin-dependent cell–cell contact. To pursue this possibility, MCF-7 cells were treated with EGTA to disrupt cell–cell contact through chelation of the extracellular calcium required for E-cadherin homophilic binding between cells. Following EGTA treatment, the cells were examined by light microscopy to document morphological changes. By 1 h of treatment with EGTA, MCF-7 cells became rounded and detached from one another. At 6 h, MCF-7 cells had no cell–cell contacts, but the cells remained attached to the culture dishes. By 18 h, cells had begun to lift from the dish and marked cell death was apparent. Cells were lysed at these various time points (excluding 18 h) and protein-normalized lysates were separated on SDS/PAGE gels. Western blotting for the LAR E-subunit revealed that LAR was expressed in high amounts at 0, 1 and 2 h, but decreased sharply after 2 h (Figure 5). After 6 h LAR protein was undetectable (results not shown). E-cadherin protein levels were also examined to determine whether loss of cell–cell contact induced by EGTA also caused changes in E-cadherin expression. E-cadherin protein was also high at 0 and 1 h, but began to decrease by 2 h (Figure 5). As a control for protein loading, cell lysates were probed for β-catenin. There was no change in β-catenin protein expression at any time point (Figure 5). Whereas both E-cadherin and LAR levels decreased with EGTA treatment, the decrease in E-cadherin commenced at an earlier time point than that of LAR. This indicates that total cellular E-cadherin does not closely correlate with LAR under these conditions. LAR did, however, decrease in response to disruption of cell–cell contacts. What is not assessed here is the impact of calcium chelation on the cellular clearance mechanisms for E-cadherin and LAR. It has been demonstrated, for example, that calcium mobilization with

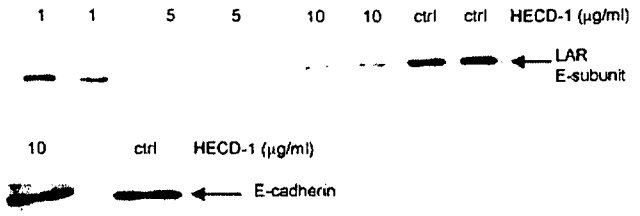

Figure 6 Effect of E-cadherin-blocking antibody on expression of LAR and E-cadherin

Confluent MCF-7 cells were treated for 24 h with media containing the E-cadherin-blocking antibody HECD-1 at the concentrations indicated (μg/ml) or the IgG control (ctrl). Cells were harvested at 24 h and LAR and E-cadherin expression were determined in cell lysates using Western blotting.

Figure 7 Effect of ectopic E-cadherin expression on density-dependent regulation of LAR

COS-7 cells were transiently transfected with constructs for LAR, E-cadherin (E-Cad) or null vector (Ctrl) as indicated. After 48 h, cells were resuspended and replated at high (lanes 1, 3, 5 and 7) or low density (lanes 2, 4, 6 and 8) for 6 h. LAR expression was determined using Western blotting.

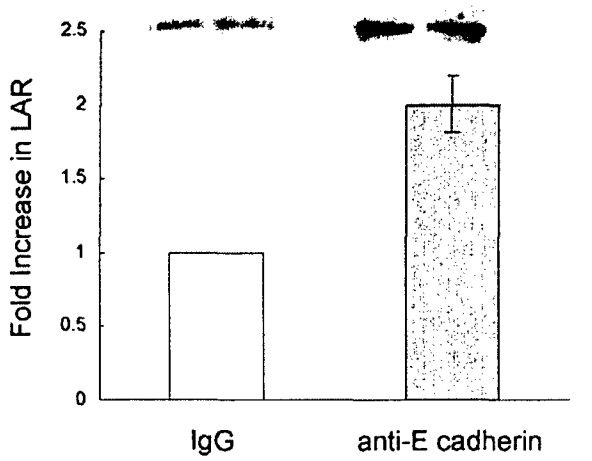

Figure 8 E-cadherin-dependent increase in LAR expression

MCF-7 cells were plated at low density on to culture dishes coated with either anti-E-cadherin antibody (HECD-1) or rabbit IgG as a control. Cells had been maintained at low density for the previous 48 h. After 12 h, cells were harvested and LAR expression determined by Western blotting. Quantitative results represent the means ± S.D. from three experiments each performed in duplicate.

ionophore A23187 induces proteolysis of cellular LAR [26]. EGTA blocks this ionophore-dependent proteolytic process. It is possible that chelation of calcium with EGTA in our experiments retarded the clearance of LAR that was otherwise initiated by the disruption and clearance of E-cadherin.

Since expression levels of LAR and E-cadherin were observed to change in parallel under most, though not all, conditions, we sought to determine whether a more direct relationship between the two proteins could be established. The homophilic cadherin-mediated cell–cell interactions are the molecular sensors of cell density. Thus we hypothesized that functional E-cadherin interactions, not necessarily total cellular E-cadherin content, may be responsible for the density-dependent change in LAR protein levels. To determine whether functional cadherin complexes control density-dependent LAR expression, we inhibited extracellular cadherin interactions directly by using an E-cadherin-specific monoclonal blocking antibody, HECD-1. Confluent MCF-7 cells were treated with 1, 5 or 10 μg/ml of HECD-1 to inhibit cell–cell contact. When the cells were analysed for morphological changes, light microscopy revealed that 5 and 10 μg/ml HECD-1 caused greater than half of the cells to round up (results not shown). When 1 μg/ml antibody was used, the cells maintained their cell–cell adhesive properties. Western blotting showed a marked decrease in LAR expression when HECD-1 was added at 5 or 10 μg/ml as compared with the control (Figure 6). Thus cadherin complexes mediate the expression of LAR protein. Under the same conditions, E-cadherin protein levels were not reduced, indicating that functional E-cadherin interactions rather than just cellular E-cadherin expression regulated LAR expression.

E-cadherin alone is not sufficient for the regulation of LAR

Since inhibition of E-cadherin homophilic interactions resulted in decreased LAR expression, we hypothesized that functional E-cadherin complexes were responsible for density-dependent regulation of LAR expression, perhaps by sequestering LAR at adherens junctions and decreasing its turnover. To examine this possibility, COS-7 cells were transiently co-transfected with mouse E-cadherin and human LAR. Transfection efficiency of E-cadherin was approx. 30–40% as assessed by immunocytochemistry. Whereas we did not have an appropriate antibody to determine the transfection efficiency of LAR, Western blotting revealed cellular expression levels of LAR that exceeded those of primary hepatocytes and several hepatoma cell lines, including HepG2 cells. It was assumed that the transfection efficiency of E-cadherin was sufficient to influence the cellular level of transfected LAR if such an interaction was occurring. While the results were variable and the data in Figure 7 show some increase in LAR levels with co-expression of E-cadherin, it was concluded that exogenous E-cadherin did not consistently alter the exogenous expression of LAR in these cells (Figure 7). Nevertheless, protein levels of exogenously expressed LAR continued to be regulated by cell density in the co-transfected COS-7 cells (see also Figure 4), suggesting that E-cadherin alone is not sufficient to regulate LAR expression.

Activation of E-cadherin complexes is sufficient to increase LAR expression

Functional E-cadherin complexes appear to be involved in the regulation of LAR expression. Perhaps ectopic expression of E-cadherin did not increase LAR levels because it did not appreciably increase the amount of functional E-cadherin complexes. To more directly investigate the role of functional E-cadherin complexes in the regulation of LAR levels, MCF-7 cells that had been maintained at low density for a minimum of 48 h were replated on culture dishes coated with either the E-cadherin antibody HECD-1 or rabbit IgG. The work of

Lambert et al. [33] has shown that interactions of cells with anti-cadherin-antibody-coated surfaces will mimic cadherin-mediated cell contact formation, recruitment of β-catenin, α-catenin and p120, and initiate cytoskeleton reorganization. Here, cells were plated at low density with little opportunity for cell–cell contact. Relative to LAR expression in control cells, LAR increased 2-fold in cells plated on the anti-E-cadherin antibody (Figure 8). Cell proliferation did not differ between the two sets of conditions during the course of the incubation.

DISCUSSION

In this study, LAR protein expression was shown to be responsive to cell density in mammary carcinoma cell lines, and functional E-cadherin complexes appeared to be the essential mediators or density sensors for this process. Additionally, transcriptional regulation did not appear to play a role in the changes in LAR expression. While the current study demonstrates that LAR levels are under the control of the E-cadherin complex, it has also been shown that LAR levels are sensitive to calcium ionophore and phorbol ester treatments [26]. Under the influence of these agents, LAR and the closely related PTPσ were shown to undergo proteolytic cleavage and subsequent shedding of their E-subunit. Loss of the E-subunit was followed by internalization of the catalytic P-subunit as shown by immunohistochemistry. The ultimate fate of the P-subunit was not determined, though P-subunit remained in the cytosol 4 h after treatment. The E-cadherin-dependent changes in LAR levels that are described in the current study with MCF-7 and Ln 3 cells indicate that comparable proportions of the P- and E-subunits are lost from cells in 4 h with a decrease in cell–cell contact. Although it cannot be ruled out that the E-subunit is being shed prior to the loss of the P-subunit, the ultimate result of the loss of cell–cell contact via decreased E-cadherin homophilic interactions is a loss of both subunits of the LAR protein. Using conditions that were similar to those used in the present study (i.e. disruption of cell–cell adhesion), Aicher et al. [26] showed that EGTA treatment of A431 cells resulted in internalization of β-catenin, plakoglobin and LAR. In this case, the entire LAR molecule was internalized (i.e. E- and P-subunits). While this internalization is assumed to lead to degradation, no comparison of the rates of loss of cellular LAR and E-cadherin were performed. Nonetheless, this report supports our conclusion that disruption of cell–cell adhesion results in removal of LAR and supports a mechanism by which the entire LAR molecule is internalized and degraded in response to a decrease in functional E-cadherin complexes.

LAR is one of several PTPs whose expression levels are influenced by cell density. We reported previously that levels of LAR, PTP1B and SHP2 (SH2-domain-containing PTP2) increase with cell density in the rat McArdle hepatoma cell line, RH7777 [24]. PTP DEP-1 (high-cell-density-enhanced PTP1) was first characterized by its increased expression and activity in high-density cultures [34]. PTPμ has been shown to be up-regulated with increasing cell density through homophilic binding between its extracellular domains expressed on adjacent cells [29]. This is not dissimilar from the mode of interaction of the cadherin family of transmembrane proteins. It has been hypothesized that PTPμ is up-regulated at high density because its homophilic interactions stabilize the PTP at the cell surface [29] and prolong its half-life. In contrast to this mechanism for stabilization of PTPs at high cell density, there is no evidence that the extracellular domain of LAR exhibits homophilic binding capability. Thus there is likely to be a mechanism other than homophilic interactions for density-dependent regulation of LAR. One report indicates, however, that one splice variant of LAR can bind to laminin–nidogen complexes via its extracellular domains [35]. This has not, however, been shown to affect the half-life of LAR in cells. Finally, our studies indicate that regulation of transcription is not an essential step in E-cadherin-mediated regulation of LAR expression. Density-dependent LAR expression is observed even when ectopic LAR expression is driven by a constitutive promoter.

Based on published data [17,23,26] and our current observations, we support a model in which LAR is sequestered in functional E-cadherin complexes by an association that stabilizes LAR and prolongs its half-life. In the absence of or in response to disruption of these E-cadherin-dependent associations, LAR is subject to a rapid turnover, as we have observed when cells are moved to low-cell-density conditions after being maintained for several days under conditions of high cell–cell contact (i.e. high density). However, our data suggest that LAR does not associate directly with E-cadherin. When both LAR and E-cadherin were ectopically expressed in COS cells, E-cadherin expression had little or no effect on the level of LAR expression. The ectopic expression of E-cadherin may not form additional functional complexes with which to sequester additional LAR. Nonetheless, the inability of E-cadherin to affect LAR levels when both are ectopically expressed in COS at low cell density indicates that E-cadherin alone is insufficient to modulate LAR levels and suggests that other components of a functional E-cadherin complex are required for the density-dependent regulation of LAR.

Whereas E-cadherin expression alone is not sufficient for modulating density-dependent increases in LAR expression, E-cadherin is necessary for this process in MCF-7 cells. This was demonstrated with cells that were plated on to a surface coated with the HECD-1 antibody. Interactions between anti-cadherin antibodies and cell-surface cadherin have been shown to mimic cell-contact formation, cadherin clustering, recruitment of the components of the activated cadherin complex and cytoskeleton rearrangement [33]. In our investigations, such antibody-mediated activation was accompanied by a 2-fold increase in LAR expression. These data demonstrate that the density-dependent increase in LAR is related directly to functional E-cadherin interactions.

Whereas LAR does not appear to associate directly with E-cadherin, evidence both *in vivo* and *in vitro* supports a direct interaction between LAR and β-catenin [17,23]. Since β-catenin is an integral component in the E-cadherin complex, along with α-catenin, plakoglobin (γ-catenin) and p120 catenin, its physical association with LAR may explain the correlation of LAR expression with functional E-cadherin complexes. Kypta et al. [17] first reported the association of LAR with β-catenin in PC12 cells and indicated that the N-terminal domain of β-catenin is necessary for this association. Neither the armadillo domains of β-catenin nor association with α-catenin are necessary for an association *in vitro*. Muller et al. [23] have shown more recently that β-catenin is a substrate for LAR *in vitro*. They observed that ectopic expression of LAR inhibits epithelial cell migration induced by EGF. This was associated with a decrease in the free, uncomplexed pool of β-catenin and in the tyrosine phosphorylation of this population of β-catenin. It has been suggested by several groups [16,36,37] that tyrosine phosphorylation of β-catenin may disrupt the interaction of the E-cadherin complex with the actin cytoskeleton. While this remains controversial, increased free β-catenin may indicate disruption of E-cadherin complexes. LAR may regulate this process, which is important to cell migration by controlling phosphorylation of β-catenin.

The data in the current study now add to this model by demonstrating that cellular levels of LAR are controlled by the

functional E-cadherin complex. Whereas stoichiometric studies were not possible, it is interesting to speculate that cellular LAR levels reflect the number of functional E-cadherin complexes. More specifically, these LAR levels may reflect β-catenin sequestration in the complexes. Muller et al. [23] have shown that free, uncomplexed β-catenin increases with EGF treatment. If this pool of free β-catenin is derived from E-cadherin complexes, might this altered localization affect the associated LAR molecule? In preliminary studies, we have observed that LAR levels decrease in response to EGF in a time course that is consistent with increases in free β-catenin (C. M. LeVea and R. A. Mooney, unpublished work).

In summary, tyrosine phosphorylation in E-cadherin complexes may be an essential regulatory mechanism to maintain cell–cell contact or to control migration in response to extracellular stimuli. Here we demonstrate that cellular levels of the transmembrane PTP LAR are regulated by functional E-cadherin complexes. The increased LAR levels at high cell density may represent a cellular mechanism to suppress further tyrosine phosphorylation of β-catenin (and plakoglobin?) in the E-cadherin complex and maintain contact inhibition of growth.

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REFERENCES

1 Gumbiner, B. M. (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell **84**, 345–357
2 Vleminckx, K. and Kemler, R. (1999) Cadherins and tissue formation: integrating adhesion and signaling. Bioessays **21**, 211–220
3 Nagafuchi, A., Ishihara, S. and Tsukita, S. (1994) The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin-α catenin fusion molecules. J. Cell. Biol. **127**, 235–245
4 Mareel, M., Bracke, M. and van Roy, F. (1994) Invasion promoter versus invasion suppressor molecules: the paradigm of E-cadherin. Mol. Biol. Rep. **19**, 45–67
5 Shiozaki, H., Oka, H., Inoue, M., Tamura, S. and Monden, M. (1996) E-cadherin mediated adhesion system in cancer cells. Cancer **77**, 1605–1613
6 Bukholm, I. K., Nesland, J. M., Karesen, R., Jacobsen, U. and Borresen-Dale, A. L. (1998) E-cadherin and alpha-, beta-, and gamma-catenin protein expression in relation to metastasis in human breast carcinoma. J. Pathol. **185**, 262–266
7 Kanner, S. B., Reynolds, A. B. and Parsons, J. T. (1991) Tyrosine phosphorylation of a 120 kD pp60src substrate upon EGF and PDGF receptor stimulation and in polyomavirus middle T-antigen transformed cells. Mol. Cell. Biol. **11**, 713–720
8 Xu, Y. and Carpenter, G. (1999) Identification of cadherin tyrosine residues that are phosphorylated and mediate Shc association. J. Cell Biochem. **75**, 264–271
9 Hinck, L., Nathke, I., Papkoff, J. and Nelsen, W. J. (1994) β-catenin: a common target for the regulation of cell adhesion by Wnt-1 and Src signaling pathways. Trends Biochem. Sci. **19**, 538–542
10 Papkoff, J. (1997) Regulation of complexed and free catenin pools by distinct mechanisms: differential effects of Wnt-1 and v-Src. J. Biol. Chem. **272**, 4536–4543
11 Takahashi, K., Suzuki, K. and Tsukatani, Y. (1997) Induction of tyrosine phosphorylation and association of β-catenin with EGF receptor upon tryptic digestion of quiescent cells at confluence. Oncogene **15**, 71–78
12 Shibata, T., Ochiai, A., Kanai, Y., Akimoto, S., Gotoh, M., Yasui, N., Machinami, R. and Hirohashi, S. (1996) Dominant negative inhibition of the association between β-catenin and c-erbB-2 by N-terminally deleted β-catenin suppresses the invasion and metastasis of cancer cells. Oncogene **13**, 883–889
13 Hiscox, S. and Jiang, W. G. (1999) Association of the HGF/SF receptor, c-met, with the cell-surface adhesion molecule, E-cadherin, and catenins in human tumor cells. Biochem. Biophys. Res. Commun. **261**, 406–411
14 Hiscox, S. and Jiang, W. G. (1999) Hepatocyte growth factor/scatter factor disrupts epithelial tumour cell-cell adhesion: involvement of beta-catenin. Anticancer Res. **19**, 509–517
15 Rahim, N., Hung, W., Tremblay, E., Sualnier, R. and Elliott, B. (1998) c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammmary carcinoma cells. J. Biol. Chem. **273**, 33714–33721
16 Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S. and Takeichi, M. (1992) Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. J. Cell Biol. **118**, 703–714
17 Kypta, R. M., Su, H. and Reichardt, L. F. (1996) Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. J. Cell. Biol. **134**, 1519–1529
18 Brady-Kalnay, S. M., Rimm, D. L. and Tonks, N. K. (1995) Receptor protein tyrosine phosphatase PTPμ associates with cadherins and catenins *in vivo*. J. Cell Biol. **130**, 977–986
19 Balsamo, J., Leung, T., Ernst, H., Zanin, M., Hoffman, S. and Lilien, J. (1996) Regulated binding of a PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of β-catenin. J. Cell. Biol. **134**, 801–813
20 Keilhack, H., Hellman, U., van Hengel, J., van Roy, F., Godovac-Zimmermann, J. and Bohmer, F. D. (2000) The protein-tyrosine phosphatase SHP-1 binds to and dephosphorylates p120 catenin. J. Biol. Chem. **275**, 26376–26384
21 Ukropec, J. A., Hollinger, M. K., Salva, S. M. and Woolkalis, M. J. (2000) SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. J. Biol. Chem. **275**, 5983–5986
22 Ozawa, M. and Kemler, R. (1998) Altered cell adhesion activity by pervanadate due to the dissociation of α-catenin from the E-cadherin-catenin complex. J. Biol. Chem. **273**, 6166–6170
23 Muller, T., Choidas, A., Reichmann, E. and Ullrich, A. (1999) Phosphorylation and free pool of β-catenin are regulated by tyrosine kinases and tyrosine phosphatase during epithelial cell migration. J. Biol. Chem. **274**, 10173–10183
24 Bleyle, L. A., Peng, Y., Ellis, C. and Mooney, R. A. (1999) Dissociatioin of PTPase levels from their modulation of insulin receptor signal transduction. Cell Signal. **11**, 719–725
25 Nose, A., Nagafuchi, A. and Takeichi, M. (1988) Expressed recombinant cadherins mediate cell sorting in model systems. Cell **54**, 993–1001
26 Aicher, B., Lerch, M. M., Muller, T., Schilling, J. and Ullrich, A. (1997) Cellular redistribution of protein tyrosine phosphatases LAR and PTPσ by inducible proteolytic processing. J. Cell Biol. **138**, 681–696
27 Schaapveld, R., Schepens, J., Robinson, G., Attema, J., Oerlemans, F., Fransen, J., Streuli, M., Wieringa, B., Hennighausen, L. and Hendriks, W. (1997) Impaired mammary gland development and function in mice lacking LAR receptor-like tyrosine phosphatase activity. Dev. Biol. **188**, 134–146
28 Neri, A., Welch, D., Kawaguchi, T. and Nicolson, G. L. (1982) Development and biological properties of malignant cell sublines and clones of a spontaneously metastasizing rat mammary adnocarcinoma. J. Natl. Cancer Inst. **68**, 507–517
29 Gebbink, M. F. B. G., Zondag, G. C. M., Koningstein, G. M., Feiken, E., Wubbolts, R. W. and Moolenaar, W. H. (1995) Cell surface expression of receptor protein tyrosine phosphatase RPTPμ is regulated by cell-cell contact. J. Cell Biol. **131**, 251–260
30 Zhang, J. S., Honkaniemi, J., Yang, T., Yeo, T. T. and Longo, F. M. (1998) LAR tyrosine phosphatase receptor: A developmental isoform is present in neurites and growth cones and its expression is regional- and cell-specific. Mol. Cell Neurosci. **10**, 271–286
31 Zhai, Y., Wirth, J., Kang, S., Welsch, C. W. and Esselman, W. J. (1995) LAR-PTPase cDNA transfection suppression of tumor growth of *neu* oncogen-transformed human breast carcinoma cells. Mol. Carcinogen. **14**, 103–110
32 Weng, L.-P., Yuan, J. and Yu, Q. (1998) Overexpression of the transmembrane tyrosine phosphatase LAR activates the caspase pathway and induces apoptosis. Curr. Biol. **8**, 247–256
33 Lambert, M., Padilla, F. and Mege, R. M. (2000) Immobilized dimers of N-cadherin-Fc chimera mimic cadherin-mediated cell contact formation: contribution of both outside-in and inside-out signals. J. Cell Sci. **113**, 2207–2219
34 Ostman, A., Yang, Q. and Tonks, N. K. (1994) Expression of DEP-1, a receptor-like PTP, is enhanced with increasing cell density. Proc. Natl. Acad. Sci. U.S.A. **91**, 9680–9684
35 O'Grady, P., Thai, T. C. and Saito, H. (1998) The laminin-nidogen complex is a ligand for a specific splice isoform of the transmembrane protein tyrosine phosphatase LAR. J. Cell Biol. **141**, 1675–1684
36 Behrens, J., Vakaet, L., Friis, R., Winterhager, E., van Roy, F., Mareel, M. M. and Birchmeier, W. (1993) Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. J. Cell Biol. **120**, 757–766
37 Kinch, M. S., Clark, G. J., Der, C. J. and Burridge, K. (1995) Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J. Cell Biol. **130**, 461–471

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EGF-dependent cell cycle progression is controlled by density-dependent regulation of Akt activation.

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The Abstract

The normal human breast epithelial cell line, MCF10A, was used to investigate the mechanism by which high-density intercellular contacts inhibit EGF-dependent cell cycle progression. EGF-dependent Akt activation was found to be transient in high-density cells and sustained in low-density cells. High-density cells also showed decreased EGF receptor (EGFR) autophosphorylation, decreased retinoblastoma protein phosphorylation, and increased p27 protein expression. Although EGFR activation was decreased in the high-density cells, the activation was sufficient to stimulate EGFR substrates comparable to low-density cells. EGF-dependent activation of the Erk1/2 pathway and the upstream activators of Akt (Gab1, erbB3, PI3 kinase, and PDK1) showed no density dependency. Antagonists of Akt activity provided further evidence that regulation of Akt activation is the critical signal transduction step controlling EGF-dependent cell cycle progression. Both adenovirus-mediated expression of dominant-negative Akt and inhibition of PI3 kinase-mediated Akt activation with LY294002 blocked cell cycle progression of low-density cells. In summary, intercellular contacts appear to block EGF-dependent cell cycle progression by inhibiting EGF signaling at the level of EGF-dependent Akt activation.

Keywords: Epidermal growth factor receptor, Akt, signal transduction, cell-cell interactions, cellular growth control, cell cycle, contact inhibition of growth.

The Introduction

Inhibition of epidermal growth factor (EGF)-dependent pathways by intercellular contacts between normal breast epithelial cells is the focus of this investigation. An elucidation of this process is relevant to understanding how breast cancers develop since mammary epithelial cells cannot form 3-dimensional tumor masses until the pathways involved in contact inhibition become dysregulated. Progressive changes in the normal breast epithelium convert the growth arrested epithelium to a malignant tumor [1]. In general, mutations which initiate neoplasia tend to be retained by more malignant subclones of the tumor [2], and, thus, loss of the signaling pathway for contact inhibition, one of the initiators of neoplasia, should be dysregulated in all subclones of the tumor. An understanding of this signaling pathway may identify a therapeutic target, which should be useful for treating *in situ*, invasive, and metastatic breast carcinomas.

Up to 30% of breast cancers overexpress one or more members of the erbB protein family. This family includes the epidermal growth factor receptor (EGFR, also known as erbB1), erbB2, erbB3, and erbB4 proteins [3]. Ligand binding causes oligomerization, tyrosine kinase activation, and erbB protein tyrosine phosphorylation [4]. Activation of EGFR, erbB2, and erbB3 cause proliferation while activation of erbB4 leads to differentiation [3, 4].

Contact inhibition of EGF-dependent signaling is thought to occur through inhibition of EGFR activation but may occur at any step in the EGF signaling pathways [3, 5-7]. One of the two major EGF-dependent pathways implicated in cellular proliferation and survival is the Erk (MAP kinase) pathway. The EGFR activates the Erk pathway following receptor autophosphorylation at tyrosine residues Y1068 and Y1086 [7]. Growth factor receptor binding protein 2 (Grb2), which is constitutively associated with son-of sevenless (SOS), binds to these

residues. SOS then activates Ras, which, in turn, activates Raf. Raf activates MAP/Erk kinase kinase (MEK) by serine phosphorylation, and MEK activates Erk by threonine and tyrosine phosphorylation [7].

The other major EGF-dependent pathway involved in EGF-dependent proliferation is the Akt pathway. The EGF-dependent activation of Akt is also initiated upon EGFR autophosphorylation [8]. Grb2, which is constitutively associated via its SH3 domain with Grb2-associated binder 1 (Gab1), binds to the EGFR. Gab1, an EGFR substrate, becomes tyrosine phosphorylated [8]. This initiates binding of the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3 kinase) to Gab1 with subsequent PI3 kinase activation [8]. PI3 kinase can also be activated via oligomerization between EGFR and erbB3 receptors [7]. Upon tyrosine phosphorylation, erbB3 binds the p85 regulatory subunit of PI3 kinase and activates the enzyme. Activation of PI3 kinase generates phosphatidylinositide-3-phosphates in the plasma membrane, which localize Akt near phosphatidylinositol-dependent kinase 1 (PDK1) [7]. Akt becomes serine/threonine phosphorylated in a PDK1-dependent manner and is activated [9].

EGF-dependent activation of Erk1/2 and Akt pathways may regulate cell cycle progression through control of p27 protein levels or by causing p27 to be sequestered away from its nuclear site of action [10-13]. P27-mediated inhibition of cell cycle progression is dosage dependent, and nuclear levels of p27 must be decreased sufficiently for cells to progress through the cell cycle. Erk1/2 phosphorylates p27 and targets it for degradation [13, 14]. Additionally, Erk1/2 activation increases cyclin D expression. Activation of Akt also decreases p27 levels through increases in cyclin D expression [13]. As Erk1/2 and Akt activation increase cyclin D expression, cyclin D sequesters p27 into the cytoplasm and releases p27-mediated inhibition of cyclin-dependent

kinase 2 (CDK2). Cyclin E binds CDK2, and cyclin E-activated CDK2 complexes phosphorylate p27 and further its degradation [13, 14]. Additionally, Akt activation blocks p27 production by inhibiting AFX/Forkhead-mediated transcription of p27 [13].

P27 is one of the proteins controlling the restriction point (R-point) of the cell cycle. The time from the beginning of G_1 to the R-point defines the time interval when cellular division is mitogen dependent. If mitogens are removed from cultures during this time interval, division ceases. After the R-point transition, the cell becomes committed to division and passes through the remaining phases of the cell cycle whether or not mitogens are present [14].

Rb is another protein that controls the R-point transition, and Rb hyperphosphorylation appears to be the critical factor determining the timing of the R-point. As cells enter the early/mid-G_1 phase of the cell cycle (when cyclin D levels are increasing, p27 levels are decreasing, and p27/CDK2 complexes are disassociating), Rb becomes partially phosphorylated by the cyclin D activated CDKs (CDK4/6) [14]. As p27 disassociates from CDK2, cyclin E binds and activates it. The R-point transition coincides with hyperphosphorylation of Rb by cyclin E-activated CDK2 [14].

P27 seems to be the key molecule integrating signals from intercellular contacts and EGF. Cell-cell contacts block EGF mitogenic signals by increasing p27 expression in mammary carcinoma cell lines grown in three-dimensional cultures [15]. As described above, inhibition of the EGF-dependent Erk1/2 and Akt pathways are potential mechanisms to increase p27 expression levels, and, thereby, inhibit cell cycle progression. Therefore, a contact inhibited cell may block cellular division by blocking one or both of the EGF-dependent Erk1/2 and Akt pathways. This would lead to high cellular p27 protein levels and low Rb phosphorylation levels.

Our results demonstrate that suppression of Akt activation is the critical mechanism of contact inhibition of EGF-dependent cell cycle progression. EGFR activation, although decreased in the high-density cells, was still sufficient to activate the Erk1/2 pathway and to tyrosine-phosphorylate erbB3 and Gab1 comparable to the low-density cells. The EGF-dependent Akt activation was transient in high-density cells. In contrast, EGF-dependent Akt activation remained elevated in the low-density cells and was required for cellular division. Low-density cells did not divide when a chemical inhibitor suppressed Akt activation or when dominant-negative Akt was introduced into the cells. This study is the first to demonstrate regulation of EGF-dependent Akt activation, rather than EGFR activation, as the critical regulatory mechanism for contact inhibition of EGF-dependent proliferation.

Materials and Methods

Materials: Anti-Akt1/PKBα (for immunoprecipitation), anti-PI3-kinase p85, anti-erbB3/HER-3, anti-Gab1 C-terminus, and anti-phosphotyrosine (clone 4G10) antibodies, and epidermal growth factor were obtained from Upstate Biotechnology. Anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), and anti-phospho-p44/42 mitogen-activated kinase (MAP kinase, Thr202/Tyr204) antibodies, and the GSK-3-α/β fusion protein substrate were from Cell Signaling Technology. Anti-EGFR (1005) (for immunoprecipitation), anti-p27 (C-19), and anti-Akt (for Western blots) antibodies were obtained from Santa Cruz Biotechnology, Inc. Anti-EGFR activated-form (for Western blots), anti-EGFR (for Western blots), and anti-β-catenin antibodies were from BD Transduction Laboratories. The anti-human retinoblastoma protein was from BD PharMingen. Anti-mouse IgG horseradish peroxidase (HRP) and anti-rabbit IgG HRP

secondary antibodies were from Promega. The protease inhibitor Cocktail Set I and cholera toxin were obtained from Calbiochem. Penicillin/streptomycin, trypsin/EDTA, and PBS were obtained from Gibco. Protein-A-Sepharose, Protein-G-Sepharose, and ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech. Dithiothreitol was purchased from Invitrogen. All other reagents were purchased from Sigma unless specifically stated.

Cell Culture: MCF10A cells were obtained from the ATCC and cultured in "complete media": DMEM/F12 media supplemented with 20 ng/ml EGF, 10 μg/ml insulin, 50 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 5% horse serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and passaged subconfluently. For density experiments, the cultures were maintained for 5 days as confluent monolayers in 10 cm dishes in order to synchronize their cell cycles. A portion of the cultures were trypsinized, replated in 15 cm dishes at 15% of their original density, and allowed to attach. After washing with PBS, the cultures were maintained for 18 hr in "starve media": DMEM/F12 media supplemented with 1% bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were treated with 5 ng/ml EGF for 0 to 30 min or 0 to 21 hr, and cellular lysates were prepared as described below.

Adenovirus Infections: Adenovirus constructs were kind gifts from Drs. Kenneth Walsh (Boston University) and Young Whang (University of North Carolina at Chapel Hill). One contained both the dominant-negative Akt and green fluorescent protein genes, and the other construct contained only the adenoviral vector control genes [16]. High-density cultures were grown as described above and infected at approximately 5 moi with either the dominant-negative Akt adenovirus or the adenovirus vector control. After 24 hr, the infected cultures were split to low-density. The cells were allowed to grow in complete media for another 24 hr before being

serum and growth factor depleted for 6 hr in starve media. Subsequently, the infected cultures were treated +/- EGF for 21hr. The cells were lifted from the dishes with trypsin/EDTA and the infected cells (expressing the green fluorescent protein) were separated from the uninfected cells (no green fluorescent protein) by fluorescence activated cell sorting. The separated cell populations were used for cell cycle analysis as described below.

Cell Cycle Analysis: DNA histograms were obtained from slides analyzed on an Oncometrics Cyto-Savant automated image cytometer. The cells were treated as described above, and then lifted from the dishes with trypsin/EDTA, cytocentrifuged onto slides, and fixed in 10% buffered formalin. Slides were stained following the protocol of Oncometrics using thionine as the DNA stain. The Cyto-Savant was programmed to scan each slide to acquire 2000 single-cell events. Debris and clumps were rejected using density and morphologic features. After acquisition, cell image galleries were reviewed to ensure only data from whole, single cells were retained in the histogram file. The calculated sum optical density (DNA index) of the cell was plotted vs. frequency (cell count).

Cellular Lysates: After treatment with 5 ng/ml EGF for the indicated time intervals, the cells were washed with ice cold PBS, lysed in ice cold buffer (100 mM HEPES pH 7.4, 150 mM NaCl, 1% triton X-100, 10% glycerol, 2.5 mM EDTA, 2 mM EGTA, 100 mM sodium fluoride, 20 mM tetrasodium pyrrophosphate, 5 mM benzamidine, 2 mM PMSF, 0.4 mM ammonium molybdate, 5 mM pervanadate, 0.5 mM AEBSF, 150 nM aprotinin, 1 μM E-64, and 1 μM leupeptin) and homogenized. The supernatants were clarified by centrifugation at 21,000 X g for 10 min at 4 °C in a Beckman Coulter Microfuge R centrifuge. Equal amounts of total cellular

protein were determined using the Bradford dye reagent according to the manufacturers protocol (Sigma).

Immunoprecipitation: To equal amounts of total cellular protein, 4 μg (for anti-erbB3 and anti-Gab1) or 5 μg (for anti-EGFR) of the immunoprecipitating antibody was added for 16 hr at 4 °C. 50 μl of a 50% w/v Protein-G-Sepharose (for erbB3 and Gab1) or 80 μl of a 50% w/v Protein-A-Sepharose (for anti-EGFR) slurry was added for 2 hr at 4 °C. The immune-complexes were washed three times with lysis buffer (see above) before loading onto a reducing SDS-PAGE gel.

Western Blot Analysis: Either total cellular lysates or immunoprecipitated proteins (as described above) were loaded onto reducing SDS-PAGE gels and transferred to nitrocellulose filters. After blocking with 5% nonfat dry milk dissolved in TBS for 30 min at 37 °C, the Western blots were probed with specific antibodies, and proteins were visualized with peroxidase coupled secondary antibodies with the ECL detection system (Amersham). The Western blots were quantitated by densitometry using the Labworks 4.0 software in the UVP BioImaging system. Subsequently, the Western blots were stripped in 67 mM Tris pH 6.8, 2% v/v SDS, 1.25 mM β-mercaptoethanol for 1 hr at 65 °C and reprobed with specific antibodies.

In Vitro Phosphatidylinositol-3-Kinase Assay: Cellular lysates were prepared as described above except that the lysis buffer did not contain ammonium molybdate; orthovanadate was substituted for pervanadate, and 1 mM dithiothreitol was added to the lysis buffer. Equal amounts of total cellular protein were immunoprecipitated with 4 μg of anti-Gab1 antibody and EGF-dependent activation of PI3 kinase was determined by an assay described previously [17]. Phosphatidylinositol-3-phosphate was separated from the reaction mixture by

thin layer chromatography, and ^{32}P incorporation was quantitated using a Storm 840 Phosphoimager from Molecular Dynamics.

In Vitro Akt Kinase Assay: Cellular lysates were prepared and Akt immunoprecipitated. The kinase assay was performed using 9 μg of GSK-3-α/β fusion protein substrate in 17 mM Tris pH 8.0, 7 mM MOPS pH 7.2, 8.0 mM β-glycerophosphate pH 7.0, 10 μCi γ-^{32}P-ATP, 167 μM ATP, 25 mM $MgCl_2$, 167 mM NaCl, 0.3 mM orthovanadate, and 0.3 mM dithiothreitol for 30 min at 30 °C. The reaction was stopped by adding EDTA to a final concentration of 400 mM and boiling for 10 min. The GSK-3-α/β fusion protein substrate was separated from the reaction mixture by non-reducing SDS-PAGE. The gels were dried, and the ^{32}P incorporated into the GSK-3-α/β fusion protein substrate was quantitated using a Storm 840 Phosphoimager by Molecular Dynamics.

Treatments with the PI3 kinase inhibitor/Akt pathway inhibitor, LY294002: Low-density cells were treated with 5 ng/ml EGF for 21 hr. After 30 min of EGF treatment, LY294002 was added to the cells to a final concentration of 30 μM. Preparation of total cellular lysates and Western blots is described above.

Results and Discussion

Contact inhibition of growth of MCF10A cells: Cell cycle progression was compared in low and high-density cells to confirm that the MCF10A cell line exhibited contact inhibition of EGF-dependent proliferation (fig. 1A). After maintaining cell cultures at confluency for five days (high-density), some of the cultures were re-seeded at low-density. The low-density cells contained no intercellular contacts (single cells) or very few intercellular contacts (small groups

of cells) (fig. 1A). High-density cells contained continuous intercellular contacts surrounding each cell's circumference (except for the cells at the periphery of the dishes) (fig. 1A). The low and high-density cells were serum and growth factor starved for 18 hr before treatment for 21 hr with a mitogenic dose of EGF (5 ng/ml). In the low-density cells, the hyperdiploid fraction (proliferative fraction: S and G2/M) increased from 22% to 58% upon EGF treatment (fig. 1A). In contrast, EGF treatment of high-density cells only increased the proliferative fraction from 16% to 20% (fig. 1A).

In addition to performing cell cycle analysis on MCF10A cells, retinoblastoma protein (Rb) phosphorylation and p27 protein levels were analyzed. The low-density cells had lower expression of the cyclin-dependent kinase inhibitor, p27, and had increased phosphorylation of the Rb protein as compared to the high-density cells (fig. 1B and 1C). As expected, in the low-density cells, p27 mass decreased upon EGF treatment. Although p27 levels also decreased in high-density cells with time of EGF treatment, the p27 levels in the high-density cells after 21 hr of EGF treatment was still higher than the p27 levels in the quiescent low-density cells (compare high-density at 21 hr to low-density at 0 hr). Together, the data in fig. 1 demonstrate that high-density MCF10A cells exhibit contact inhibition of EGF-dependent cell cycle progression and that p27 protein levels and Rb phosphorylation levels represent molecular markers of cell cycle progression.

The partial Rb phosphorylation observed in the high-density cells is not surprising (fig. 1B). Previous studies [13, 14] have shown that mitogens, such as EGF, can cause phosphorylation of Rb by cyclin D-activated CDK4/6. However, this Rb phosphorylation is not enough to drive cells through the cell cycle. Therefore, both the EGF-dependent partial

phosphorylation of Rb and the inhibition of cell cycle progression seen in high-density MCF10A cells is expected and supported by the literature [13, 14].

The decrease in p27 expression under both density conditions was also expected. It has been shown [13, 14] that EGF treatment increases cyclin D expression through activation of Erk and Akt. Once activated, Erk phosphorylates p27 and targets it for degradation. Cyclin D can sequester p27 in the cytoplasm, functionally inactivating it. Additionally, Akt activation can inhibit p27 transcription. Therefore, if Erk1/2 and Akt become activated in high-density cells, then one or both of these pathways may decrease p27 expression [13]. It is the magnitude of the decreased nuclear p27 that is important since p27-mediated inhibition of CDK2 exhibits a dosage effect. A 50% decrease in protein expression will render p27 unable to inhibit sufficient amounts of CDK2 to block cellular proliferation [13]. The residual p27 expression in the low-density cells at 21 hr has been decreased by more than 50% (fig. 1C), and, thus, cannot block cellular division (fig. 1A).

Analysis of EGFR activation: EGFR activation was compared in low and high-density cultures as a starting point in determining the steps in the EGF-dependent signaling pathways that are regulated by intercellular contacts. The cultures were grown to confluent monolayers in order to synchronize their cell cycles. Subsequently, some of the cultures were split to low-density. Both densities were serum and growth factor starved for 18 hr and treated with 5 ng/ml EGF for 0 to 30 min. The 30 min time interval was chosen to ensure that any differences in EGF signaling would be an acute response to intercellular contacts and not to density-dependent differences in transcription or translation.

Western blot analysis of whole cell lysates with an antibody that recognizes the tyrosine-phosphorylated form of the EGFR (fig 2A) demonstrated greater EGFR autophosphorylation in low-density cells than in high-density cells. This indicates that EGFR in the low-density cells were activated to a greater extent than in high-density cells at all time points examined (fig. 2A).

A 6% SDS-PAGE gel allows separation of EGFR into separate migrating forms. Under these conditions, slower (tyrosine phosphorylated - activated) and faster (basal – inactive) migrating forms are resolved. The low-density cells had more EGFR in the slower migrating form, which represents the tyrosine-phosphorylated state of the receptor (fig. 2B). The data in fig. 2A, which estimate the activated EGFR, suggest a more marked difference in receptor activation between the two density conditions than does the same data when analyzed by the differential electrophoretic migration method (fig. 2B). However, similar conclusions can be drawn from both parts of fig. 2: EGFR in the high-density cells are less activated, but a measurable steady state level of EGFR activation was present in these cells upon EGF treatment. There was also a trend towards higher total EGFR mass in the low-density cells (fig. 2B), but this increase was not significant when three separate experiments were compared.

This study is in agreement with others showing EGFR to be more active in low-density cells than in high-density cells [5, 6, 18, 19]. These density-dependent differences in EGFR activation have been correlated with density-dependent differences in EGFR localization and tyrosine phosphatase activation. Low-density cells contain EGFR that are homogenously dispersed over the plasma membrane, and EGFR in high-density cells are restricted to regions of intercellular contacts [18, 20]. Increased tyrosine phosphatase activation in high-density cells has been proposed as the mechanism of contact inhibition of growth [5, 6]. Although our data are

consistent with the fact that EGFR activation in high-density cells is limited, perhaps, by the increased tyrosine phosphatase activation in these cells, the following data will show that the ability of EGFR to signal to their substrates has not been limited. Also, the data to be presented will argue for inhibition of a step other than the EGFR as the critical mechanism of contact inhibition of EGF-dependent growth.

Analysis of EGF-dependent Akt activation: EGF-dependent Akt activation was examined to determine if suppression of the EGFR in high-density cells has any impact on downstream EGF-dependent pathways. The phosphorylation specific Akt antibody, phosphoserine 473 (pSer473-Akt), was used to assess Akt activation (fig. 3A). In contrast to the decreased EGFR activation observed at all time points in the high-density cells, EGF similarly activated Akt at 5 min and 10 min in both high and low-density cells. After 10 min, in contrast to the low-density cells, Akt activation markedly decreased by 60-70% in the high-density cells (fig 3A and 3D). Akt activation remained relatively constant throughout the 30 min time course (only decreasing by 10-20%) in the low-density cells (fig 3D). The mass of Akt was similar under both density conditions (fig. 3B), and β-catenin (used as a loading control) showed no difference under the high and low-density conditions (fig. 3C). These results indicate that Akt activation as well as EGFR activation in high-density cells was decreased, but the time course of suppression of Akt and EGFR activities differ. At this point in our experiments, it was unclear if the suppressed EGF-dependent Akt activation in the high-density cells was simply a direct reflection of the decreased EGFR activation in these cells. Or, an alternative explanation, which the rest of our experiments will demonstrate, is that the suppressed Akt activation was the mechanism responsible for contact inhibition of EGF-dependent growth in these cells.

Analysis of EGF-dependent Erk1/2 activation: EGFR activation is suppressed in high-density cells relative to low-density cells; it would be predicted that all EGF-dependent signals downstream of the EGFR should be inhibited relative to the low-density cells. To test this hypothesis, EGF-dependent Erk1/2 activation was examined. As seen in fig. 4A, Erk1 (the higher molecular weight band) was activated under the high-density condition to a slightly greater extent than under the low-density condition. However, this increased activation was not statistically significant when three separate experiments were compared. This activation of Erk1 occurred in the high-density cells even though the EGFR in these cells were less activated (fig. 2A). Similarly, EGF-dependent Erk2 activation was somewhat higher in the high-density cells at 5 and 10 min (fig. 4B), but this increase was not statistically significant when three experiments were compared. Erk1/2 masses were similar at both densities (fig. 4C).

Analysis of the EGFR substrates, Gab1 and erbB3, and their ability to activate PI3 kinase: Although suppressed relative to low-density cells, the magnitude of EGFR activation in high-density cells appears sufficient to fully activate the EGF-dependent Erk1/2 pathway (fig. 4). Why does density-dependent suppression of EGFR activity leave the EGF-dependent Erk1/2 pathway unaffected while suppressing the EGF-dependent Akt pathway? We examined the tyrosine-phosphorylation states of EGFR substrates involved in Akt activation, Gab1 and erbB3, to begin to answer that question. Both Gab1 and erbB3 show EGF-dependent increases in tyrosine phosphorylation (fig. 5A and 5D, respectively). The Gab1 tyrosine phosphorylation was maximal by 5 min and had similar kinetics under both culture conditions (fig. 5A). The EGF-stimulated erbB3 tyrosine phosphorylation was maximal by 5 min, and remained essentially unchanged under both density conditions throughout the EGF time course (fig. 5D). Gab1 and erbB3 masses

were similar under the high and low-density conditions (fig. 5B and 5E). These results indicate that the decreased EGF-dependent Akt activation in high-density cells is not simply a direct reflection of the decreased EGFR activation in these cells. The lower steady state EGFR activation in the high-density cells does not limit signaling through the Erk1/2 pathway or to Gab1 and erbB3. Therefore, the critical step inhibiting EGF-dependent Akt activation must be downstream from the EGFR somewhere between Gab1/erbB3 and Akt.

Following tyrosine phosphorylation of Gab1 and erbB3, the next step in the EGF-dependent activation of Akt is PI3 kinase activation. PI3 kinase is activated through association of its p85 subunit with phosphotyrosine residues on erbB3 and Gab1 [7, 8]. Do high-density intercellular contacts inhibit Akt activation by inhibiting PI3 kinase activation? Gab1 and erbB3 were immunoprecipitated, and the amounts of p85 associated with these proteins were determined by Western blot analysis. Similar levels of p85 were associated with Gab1 in the low and high-density cells (fig. 5C). ErbB3 from high-density cells had a somewhat larger amount of associated p85 in the basal state (fig. 5F). Nonetheless, EGF treatment resulted in comparable amounts of erbB3-associated p85 at both densities. These results argue that the observed differences in Akt activation between high and low-density cells cannot be explained by differences in PI3 kinase association with upstream activators.

Analysis of in vitro PI3 kinase activation: The Gab1-associated PI3 kinase activation was measured by an *in vitro* kinase assay to confirm that the amount of p85 subunit associated with Gab1 reflects PI3 kinase enzymatic activity. No difference in Gab1-associated PI3 kinase activation was observed between the low and high-density cells (fig. 6). The Gab1-associated PI3 kinase activation was maximal at 5 min and decreased by 40% at 30 min (fig. 6B). Western

blots of p85 subunit association with Gab1 paralleled *in vitro* PI3 kinase activation (fig. 5C and fig. 6), and, thus, p85 co-immunoprecipitation assays are an accurate representation of PI3 kinase activation in MCF10A cells.

Analysis of the 3-Phosphoinositide-dependent kinase-1 phosphorylation (activation) of Akt: Despite differences in EGF-dependent Akt activation between low and high-density cells, EGF-dependent tyrosine phosphorylation of Gab1 and erbB3, and the subsequent activation of PI3 kinase under these two conditions were essentially identical. Regulation of Akt activation would appear to be at a step below PI3 kinase activation. The serine/threonine kinase PDK1 is positioned immediately downstream of PI3 kinase and activates Akt by phosphorylating Akt on threonine 308 [9]. Therefore, a phosphorylation specific antibody, phosphothreonine 308 Akt (pThr308Akt), was used to examine whether high-density intercellular contacts regulate PDK1-mediated activation of Akt.

EGF treatment led to comparable phosphorylation of threonine 308 on Akt in both high and low-density cells (fig. 7A). Phosphorylation of Akt threonine 308 decreased with length of EGF treatment and had similar kinetics in high and low-density cells (fig. 7A). No significant differences were observed in pThr308Akt phosphorylation when three separate experiments were compared. Therefore, PDK1 activates Akt, similarly, under both density conditions.

Analysis of in vitro Akt kinase activation: High-density intercellular contacts interfere with sustained activation of Akt as evidenced by the decreased pSer473-Akt in the high-density cells (fig. 3). *In vitro* Akt kinase assays were performed to confirm that the observed difference in phosphorylation of serine 473 on Akt reflects differences in enzymatic activation. The ability of immunoprecipitated Akt to phosphorylate a soluble glycogen synthase kinase-3-α/β (GSK-3-

α/β) fusion protein was determined (fig. 8). The low-density cells had higher basal and EGF-stimulated Akt activities (fig. 8A). At 5 and 30 min, these differences were statistically significant ($p \leq 0.05$, n=3) (fig. 8B). In the low-density cells, the *in vitro* Akt kinase activation remaining at 30 min was greater than the maximal Akt activation attained by the high-density cells (fig. 8). Comparable amounts of Akt were in the low and high-density immunoprecipitates when assessed by Western blot analysis.

Analysis of Akt activation during cell cycle progression: Initially, only the early time intervals after EGF treatment were investigated. This was performed in order to determine the acute effects of high-density intercellular contacts on EGF signaling. Does the difference in EGF-dependent Akt activation during these early time intervals remain over the time required for cell cycle progression? To answer this question, differences in phosphorylation of Akt on serine 473 were examined over a 21 hr time interval. At all time points tested, the low-density cells had higher Akt activation (fig. 9A). Therefore, high-density intercellular contacts suppress Akt activation by 20 min, and this activation remains decreased for 21 hr (fig. 3 and fig. 9).

Suppression of Akt activation in low-density cells prevents cell cycle progression: EGF-dependent Akt activation in high-density cells was transient, but it remained sustained in low-density cells. Is sustained EGF-dependent Akt activation necessary for EGF-dependent proliferation? Will low-density cells divide if EGF-dependent Akt activation were rendered transient? Akt was activated in low-density cells by treatment with 5 ng/ml EGF for 30 min. Subsequently, Akt activation was suppressed by supplementing the media with 30 μM LY294002, a PI3 kinase inhibitor, which suppresses PI3 kinase-mediated Akt activation. (Initial experiments demonstrated that 30 μM LY294002 decreases phosphorylation of Akt serine 473 in

low-density cells to levels similar to high-density cells after 30 min of EGF treatment. The EGF-dependent activation of Erk1/2 was unaffected by LY294002. Data not shown.) After 21 hr of EGF treatment +/- LY294002, the cell cycle progression indices, Rb and p27, were analyzed. As expected, EGF treatment of low-density cells increased Akt activation, caused Rb hyperphosphorylation, and decreased p27 levels when compared to untreated cells (fig. 10). LY294002 suppressed the EGF-dependent Akt activation, almost to the basal state, and prevented basal Rb phosphorylation. The effects on Rb phosphorylation are most likely a consequence of the effects of PI3 kinase inhibition on other pathways, in addition to the Akt pathway, which affect the Rb Phosphorylation State. Additionally, LY294002 treatment prevented the EGF-dependent decrease in p27 levels, and the p27 levels at 21 hr remained similar to the quiescent state (fig 10).

Akt activation is necessary for cell cycle progression: Low-density cells, which had been infected with an adenovirus expressing both dominant-negative Akt and green fluorescent protein [16] or with an adenovirus containing only control genes, were treated +/- EGF for 21 hr. Subsequently, the cells were separated by fluorescence activated cell sorting to isolate the dominant-negative Akt infected cells (expressing green fluorescent proteins). The dominant-negative Akt infected cells, control adenovirus infected cells, and uninfected cells were subjected to cell cycle analysis. As can be seen in fig 11, EGF stimulated the proliferative fraction (S and G2/M) in the uninfected cells from 19% to 44% and in the adenovirus vector control infected cells from 28% to 45%. However, the dominant-negative Akt expressing cells were blocked from EGF-dependent cell cycle progression. They showed a proliferative fraction that only

increased from 16% to 27%. The comparison among the three conditions demonstrates that EGF-dependent Akt activation is necessary for cell cycle progression.

Intercellular contacts suppress the full activation of Akt by suppressing phosphorylation of serine 473 (fig. 3). Three mechanisms have been proposed to explain the modulation of Akt activation on serine 473 [9, 21]. First, a putative kinase distinct from PDK1, termed PDK2, may directly phosphorylate Akt on serine 473, fully activating the kinase. Second, Akt may become fully activated when a C-terminal fragment of protein kinase C-related kinase 2 interacts with PDK1. This interaction may shift PDK1's substrate specificity away from threonine 308 to serine 473 [9]. Third, PDK1-mediated phosphorylation of Akt on threonine 308 may permit Akt to auto-activate by phosphorylating itself on serine 473. Our data shows PDK1-mediated phosphorylation of Akt on threonine 308 to be similar at both cell densities (fig. 7). If the third mechanism (above) occurred in MCF10A cells, then one would predict that phosphorylation of Akt on serine 473 should also be similar at both cell densities. This was not observed in our experiments (fig. 3). Thus, only the first two mechanisms of Akt activation (above) are compatible with our data.

In addition to regulation by serine and threonine phosphorylation, Akt is regulated by tyrosine phosphorylation [22, 23]. EGF treatment induces tyrosine phosphorylation of Akt in COS1 cells [22]. This EGF-dependent tyrosine phosphorylation of Akt can be inhibited by PP2, a selective inhibitor of Src family tyrosine kinases. Recently, Akt has been shown to be phosphorylated on tyrosine 474 in COS1 cells treated with pervanadate, serum, or insulin-like growth factor 1 (IGF-1) [23]. This tyrosine phosphorylation was required for full activation of Akt by pervanadate and IGF-1. When tyrosine 474 was replaced by a phenylalanine, a 55%

decrease in pervanadate and IGF-1 stimulated Akt activation was observed [23]. Therefore, tyrosine phosphorylation/dephosphorylation is also a possible mechanism by which cell density may regulate Akt activation. We have yet to test this possibility.

High-density may regulate Akt activation by increasing serine/threonine dephosphorylation. Phosphatase 2A inhibits Akt activation by dephosphorylating both phosphothreonine 308 in the Akt activation loop and phosphoserine 473 in its C-terminus [9]. Future experiments will be needed to test this potential mechanism.

Other studies support our conclusion that Akt activation, and not Erk1/2 activation, plays a critical mitogenic role for breast cancer cell lines [24, 25]. Using synthetic inhibitors of the Erk1/2 pathway, PD098059, and the PI3 kinase/Akt pathway, LY294002, Dufourny *et al.* showed that IGF-1 mediated division in MCF-7 cell cultures was dependent on PI3 kinase/Akt activation and independent of Erk1/2 activation [24]. In a separate study, Busse *et. al.* used a quinazoline inhibitor of the EGFR kinase in MDA-468 breast carcinoma cells to induce growth arrest [25]. This result could be reproduced by blocking the PI3 kinase/Akt pathway, but growth arrest did not occur if only the Erk1/2 pathway was blocked [25]. These studies, together with ours, argue for a critical role of Akt, not Erk1/2, in the regulation of cell cycle progression of breast epithelial cells.

Our data argue that a sustained EGF-dependent Akt activation is required for low-density cells to divide and are in agreement with other studies linking sustained Akt activation to regulation of proliferation. Sustained α-thrombin-induced Akt activation in Chinese hamster embryonic fibroblasts was required for DNA synthesis [26], and sustained activation of Akt was

required for proliferation of pancreatic β-cells [27]. If Akt was only transiently activated, proliferation was not observed [27].

How does suppression of Akt activation in high-density cells cause growth arrest? The most likely mechanism involves Akt-dependent regulation of p27 expression levels, although inhibition of the nuclear localization of p27 may also be involved. Decreased Akt activation in high-density cells would be predicted to lead to increased p27 levels [13, 14]. If p27 expression levels remain above a critical level, 50% of maximum, division will not occur [13, 14]. Since our data shows that EGF activates Erk1/2 in high-density cells but they do not divide, Erk1/2 activation by itself is not sufficient to lower p27 below the level critical to allow proliferation. Thus, low-density cells seem to require both EGF-dependent Erk1/2 and Akt activation to decrease p27 levels sufficiently to allow division. Intercellular contacts appear to function as a rheostat modulating Akt activation, thus, controlling the ability of a cell to withdraw from or enter the cell cycle.

The molecular composition of this rheostat may be E-cadherin homophilic intercellular bridges. Increases in E-cadherin-mediated intercellular contacts have been associated with up-regulation of p27 protein levels [15], but the exact mechanism up-regulating p27 expression has not been determined. A mechanism involving E-cadherin-mediated regulation of Akt phosphorylation on serine 473 would be predicted by our data.

This study is the first to report that contact inhibition of EGF-dependent proliferation occurs at the level of Akt activation rather than at the level of EGFR activation. Although we have observed inhibition of EGFR activation in high-density cells, this inhibition does not affect signaling immediately downstream of the EGFR or at the level of EGF-dependent Erk1/2

activation. Therefore, suppression of EGFR activation is not the primary contributor to contact inhibition under our conditions. Future efforts will be directed towards an understanding of the mechanism by which Akt activation is regulated by intercellular contacts.

Acknowledgements

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References

1. Liotta, L. A., and Kohn, E. C. (2000). Invasion and Metastases. *In* "Cancer Medicine" B.C. Decker, Hamilton, Ontario.
2. Nowell, P. C. (1989). Chromosomal and Molecular Clues to Tumor Progression. *Semin Oncol* **16,** 116-127.
3. Yarden, Y., and Sliwkowski, M. X. (2001). Untangling the ErbB signaling network. *Nat.Rev.Mol.Cell Biol.* **2,** 127-137.

4. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* **19,** 3159-3167.

5. Mansbridge, J. N., Knuchel, R., Knapp, A. M., and Sutherland, R. M. (1992). Importance of tyrosine phosphatases in the effects of cell-cell contact and microenvironments on EGF-stimulated tyrosine phosphorylation. *J.Cell Physiol* **151,** 433-442.

6. Sorby, M., and Ostman, A. (1996). Protein-tyrosine phosphatase-mediated decrease of epidermal growth factor and platelet-derived growth factor receptor tyrosine phosphorylation in high cell density cultures. *J.Biol.Chem.* **271,** 10963-10966.

7. Schmidt, M., and Lichtner, R. B. (2002). EGF receptor targeting in therapy-resistant human tumors. *Drug Resist.Updat.* **5,** 11-18.

8. Liu, Y., and Rohrschneider, L. R. (2002). The gift of Gab. *FEBS Lett.* **515,** 1-7.

9. Kandel, E. S., and Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp.Cell Res.* **253,** 210-229.

10. Delmas, C., Manenti, S., Boudjelal, A., Peyssonnaux, C., Eychene, A., and Darbon, J. M. (2001). The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells. *J.Biol.Chem.* **276,** 34958-34965.

11. Hoshino, R., Tanimura, S., Watanabe, K., Kataoka, T., and Kohno, M. (2001). Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27 (Kip1). *J.Biol.Chem.* **276,** 2686-2692.

12. Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K., Allen, S., Paul, J. D., Hbaiu, A., Goode, R. G., Sandusky, G. E., Vessella, R. L., and Neubauer, B. L. (2000). Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J.Biol.Chem.* **275,** 24500-24505.

13. Philipp-Staheli, J., Payne, S. R., and Kemp, C. J. (2001). p27 (Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Exp.Cell Res.* **264,** 148-168.

14. Planas-Silva, M. D., and Weinberg, R. A. (1997). The restriction point and control of cell proliferation. *Curr.Opin.Cell Biol.* **9,** 768-772.

15. St Croix, B., Sheehan, C., Rak, J. W., Florenes, V. A., Slingerland, J. M., and Kerbel, R. S. (1998). E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27 (KIP1). *J.Cell Biol.* **142,** 557-571.

16. Morales-Ruiz, M., Fulton, D., Sowa, G., Languino, L. R., Fujio, Y., Walsh, K., and Sessa, W. C. (2000). Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ Res* **86,** 892-6.

17. Way, B. A., and Mooney, R. A. (1993). Activation of phosphatidylinositol-3-kinase by platelet-derived growth factor and insulin-like growth factor-1 is inhibited by a transmembrane phosphotyrosine phosphatase. *J Biol Chem* **268,** 26409-15.

18. Lichtner, R. B., and Schirrmacher, V. (1990). Cellular distribution and biological activity of epidermal growth factor receptors in A431 cells are influenced by cell-cell contact. *J.Cell Physiol* **144,** 303-312.

19. Takahashi, K., and Suzuki, K. (1996). Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell-cell adhesion. *Exp.Cell Res.* **226,** 214-222.

20. Suarez-Quian, C. A., and Byers, S. W. (1993). Redistribution of epidermal growth factor receptor as a function of cell density, cell-cell adhesion and calcium in human (A-431) cells. *Tissue Cell* **25,** 1-17.

21. Wick, M. J., Dong, L. Q., Riojas, R. A., Ramos, F. J., and Liu, F. (2000). Mechanism of phosphorylation of protein kinase B/Akt by a constitutively active 3-phosphoinositide-dependent protein kinase-1. *J.Biol.Chem.* **275,** 40400-40406.

22. Chen, R., Kim, O., Yang, J., Sato, K., Eisenmann, K. M., McCarthy, J., Chen, H., and Qiu, Y. (2001). Regulation of Akt/PKB activation by tyrosine phosphorylation. *J.Biol.Chem.* **276,** 31858-31862.

23. Conus, N. M., Hannan, K. M., Cristiano, B. E., Hemmings, B. A., and Pearson, R. B. (2002). Direct identification of tyrosine 474 as a regulatory phosphorylation site for the akt protein kinase. *J.Biol.Chem.* **277,** 38021-38028.

24. Dufourny, B., Alblas, J., van Teeffelen, H. A., van Schaik, F. M., van der, B. B., Steenbergh, P. H., and Sussenbach, J. S. (1997). Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-

kinase and is independent of mitogen-activated protein kinase. *J.Biol.Chem.* **272,** 31163-31171.

25. Busse, D., Doughty, R. S., Ramsey, T. T., Russell, W. E., Price, J. O., Flanagan, W. M., Shawver, L. K., and Arteaga, C. L. (2000). Reversible G(1) arrest induced by inhibition of the epidermal growth factor receptor tyrosine kinase requires up-regulation of p27 (KIP1) independent of MAPK activity. *J.Biol.Chem.* **275,** 6987-6995.

26. Goel, R., Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2002). alpha-Thrombin induces rapid and sustained Akt phosphorylation by beta- arrestin1-dependent and -independent mechanisms, and only the sustained Akt phosphorylation is essential for G1 phase progression. *J.Biol.Chem.* **277,** 18640-18648.

27. Lingohr, M. K., Dickson, L. M., McCuaig, J. F., Hugl, S. R., Twardzik, D. R., and Rhodes, C. J. (2002). Activation of IRS-2-mediated signal transduction by IGF-1, but not TGF- alpha or EGF, augments pancreatic beta-cell proliferation. *Diabetes* **51,** 966-976.

Legends to Figures

Fig. 1. High-density MCF10A cells do not progress through the cell cycle. MCF10A cells were grown to confluence (high-density), and then half of the cultures were reseeded at 15% confluence (low-density). The cells were placed in growth factor free media for 18 hr and subsequently treated for 21 hours (**A**) or the times indicated (**B, C**) with 5 ng/ml EGF. **A) Top panels** show low and high-density cell cultures at 100X. The cells were processed for cell cycle analysis as described in the *Materials and Methods.* DNA histograms were obtained from slides analyzed on an Oncometrics Cyto-Savant automated image cytometer. DNA content was

measured according to the Oncometrics protocol using thionine as the DNA stain. The calculated sum optical density (DNA index) was plotted vs. frequency (cell count). **Middle panels** show the cell cycle profiles of low and high-density cells under growth factor starved conditions. **Lower panels** show the cell cycle profiles of low and high-density cells after 21 hr of treatment with 5 ng/ml of EGF. (**B, C**) Using equal amounts of cell lysates, **B)** phosphorylated Rb (slower migrating band) and hypophosphorylated Rb (faster migrating band) and **C)** p27 expression were assessed by Western blot analysis.

Fig. 2. EGF receptor activation is decreased in high-density cells. MCF10A cells were treated as described in fig. 1. **A)** The activated, tyrosine phosphorylated EGFR (PY-EGFR) and **B)** EGFR expression (EGFR) was assessed by Western blots (IB) analysis under high and low-density conditions. These are representative blots from 3 separate experiments.

Fig 3. EGF-dependent Akt activation is transient in high-density cells and sustained in low-density cells. MCF10A cells were treated as described in fig. 1. **A)** Western blot (IB) analysis of phosphoserine 473 Akt (pSer473-Akt), and **B)** Akt protein expression (Akt) are shown under high and low-density conditions as a function of time after EGF treatment. In **C)**, β-catenin is used as a loading control. In **D)** data are plotted as percentage of maximum serine 473 Akt phosphorylation as a function of time. Each point represents the average +/- one standard deviation from densitometry scans of 3 separate experiments. * Designates statistically significant differences between the two densities, $p \leq 0.05$ by t-test.

Fig. 4. In high-density cells EGF-dependent activation of Erk1/2 is not suppressed in comparison to the low-density cells. MCF10A cells were treated as described in fig. 1. **A)** Phosphorylated Erk1/2 (pErk1/2) was assessed by Western blot (IB) analysis. **B)** To more effectively examine Erk2, a shorter exposure was used. **C)** Erk1/2 protein expression (Erk1/2) was also assessed by Western blot analysis. These are representative blots from 3 separate experiments.

Fig. 5. EGF-dependent tyrosine phosphorylation of Gab1 and erbB3, and their association with p85 subunit of PI3 kinase are comparable in high and low-density cells. MCF10A cells were treated as described in fig. 1. Equal amounts of protein in total cellular lysates were immunoprecipitated (IP) with **(A, B, C)** anti-Gab1 (Gab1) or **(D, E, F)** anti-erbB3 antibodies. Western blots (IB) were then probed with antibodies to **(A, D)** phosphotyrosine (PY), **B)** Gab1 (Gab1), **(C, F)** p85 subunit of PI3 kinase (p85), and **E)** erbB3 (erbB3).

Fig. 6. The EGF-dependent Gab1-associated *in vitro* PI3 kinase activation is similar in low and high-density cells. MCF10A cells were treated as described in fig. 1. Equal amounts of protein in total cellular lysates were immunoprecipitated (IP) with anti-Gab1 antibody (Gab1) and analyzed for associated PI3 kinase activation as described in the *Materials and Methods*. **A)** A representative autoradiogram of ^{32}P-Phosphatidylinositol-3-phosphate (PIP3) is shown. **B)** Data from 3 separate experiments are plotted as a function of maximum PI3-kinase activity. Each point represents the average +/- one standard deviation from phosphoimager analysis.

Fig 7. The EGF-dependent 3-phosphoinositide-dependent kinase-1 phosphorylation (activation) of Akt. MCF10A cells were treated as described in fig. 1. **A)** Western blot (IB) analysis of phosphothreonine 308 Akt (pThr308Akt), and **B)** Akt protein expression (Akt) are shown under high and low-density conditions as a function of time after EGF treatment. These are representative blots from 3 separate experiments.

Fig. 8. The EGF-stimulated *in vitro* kinase activation of Akt is decreased in high-density cells. MCF10A cells were treated as described in fig. 1. After immunoprecipitating with anti-Akt1, *in vitro* phosphorylation of glycogen synthase kinase-3-α/β fusion proteins (GSK-3-α/β) was analyzed. **A)** A representative autoradiogram of ^{32}P-GSK-3-α/β is shown at 3 time points. **B)** Data from 3 separate experiments are plotted as a function of GSK phosphorylation. Each point represents the average +/- one standard deviation from phosphoimager analysis. The data are expressed as arbitrary units (U). * Designates statistically significant differences between the two densities, $p \leq 0.05$ by t-test.

Fig. 9. EGF-dependent Akt activation remains suppressed in high-density cells during long-term EGF stimulation. MCF10A cells were treated as described in fig. 1, and equal amounts of protein in total cellular lysates were examined by Western blot (IB) analysis for **A)** phosphoserine 473 Akt (pSer473-Akt), and **B)** Akt mass (Akt). (Part **A** was overexposed in order to show pSer473-Akt in the high-density cells.)

Fig. 10. Inhibition of PI3 kinase mediated Akt activation blocks cell cycle progression in low-density cells. Low-density cells were treated for 21 hr with or without 5 ng/ml of EGF. After an initial 30 min of EGF treatment, the cells were further treated +/- 30 μM LY294002. Protein normalized lysates were separated by SDS-PAGE. **A)** Western blot analysis of phosphoserine 473 Akt (pSer473-Akt), **B)** retinoblastoma protein (Rb) phosphorylation, and **C)** p27 mass (p27) are shown.

Fig. 11. Adenovirus mediated expression of dominant-negative Akt prevents cell cycle progression in low-density cells. Low-density cells were infected with adenovirus containing dominant-negative Akt or control adenoviral DNA. These infected cells plus uninfected control cells were treated +/- 5 ng/ml EGF for 21 hr and processed for cell cycle analysis as described in the *Materials and Methods*. DNA histograms were obtained from slides analyzed on an Oncometrics Cyto-Savant automated image cytometer. DNA content was measured according to the Oncometrics protocol using thionine as the DNA stain. The calculated sum optical density (DNA index) was plotted vs. frequency (cell count).

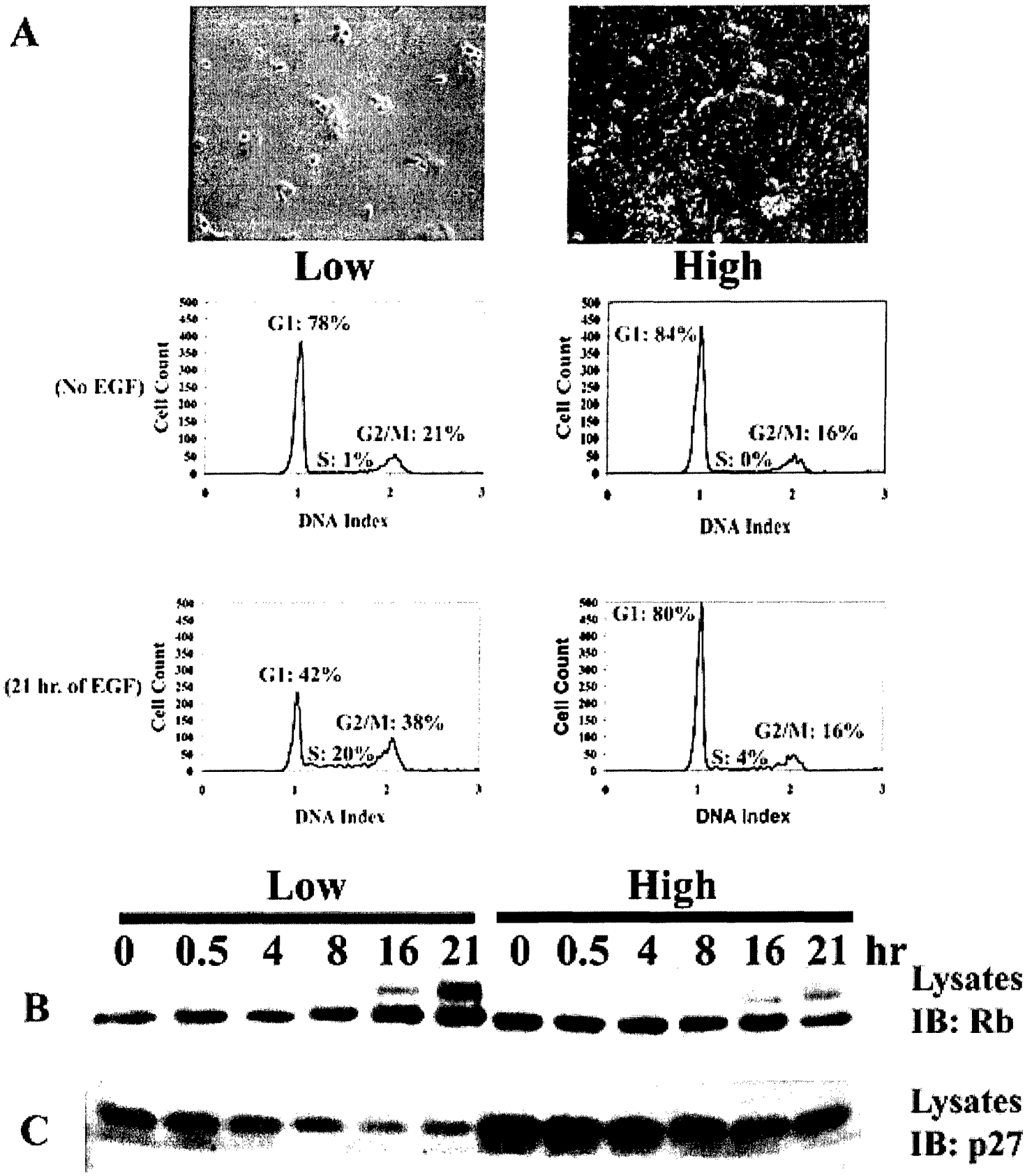

A
Low
High
(No EGF)
G1: 78%
G2/M: 21%
S: 1%
G1: 84%
G2/M: 16%
S: 0%
Cell Count
DNA Index
(21 hr. of EGF)
G1: 42%
G2/M: 38%
S: 20%
G1: 80%
G2/M: 16%
S: 4%
Low
High
0 0.5 4 8 16 21 0 0.5 4 8 16 21 hr
B
Lysates
IB: Rb
C
Lysates
IB: p27

fig. 1

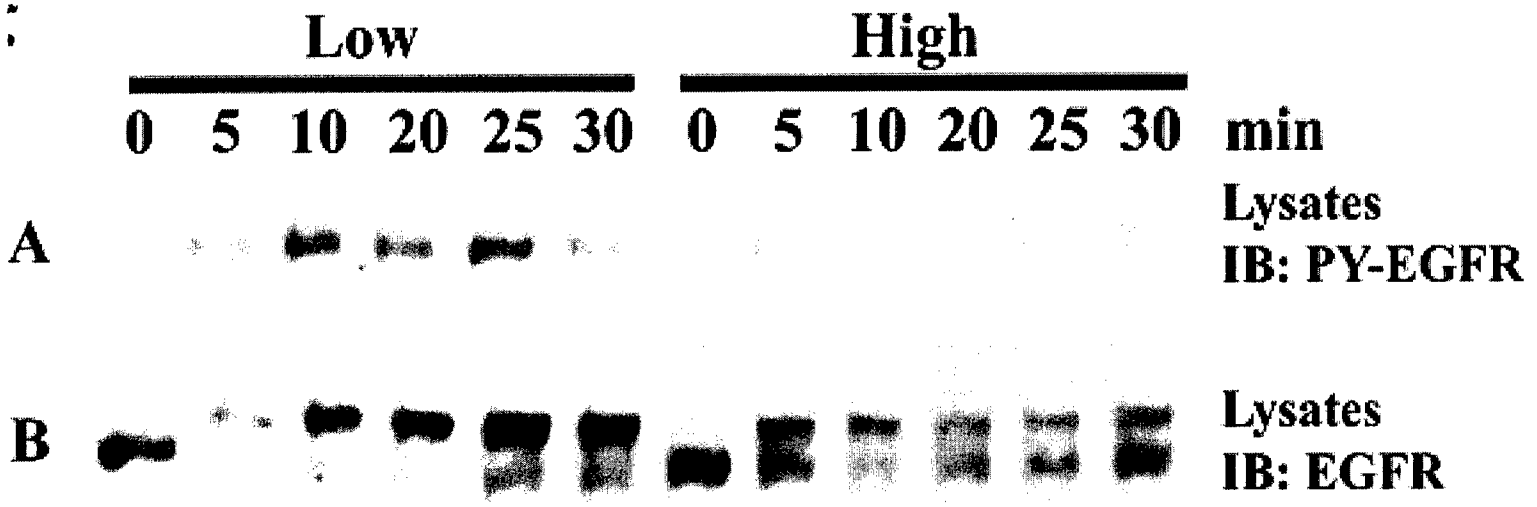
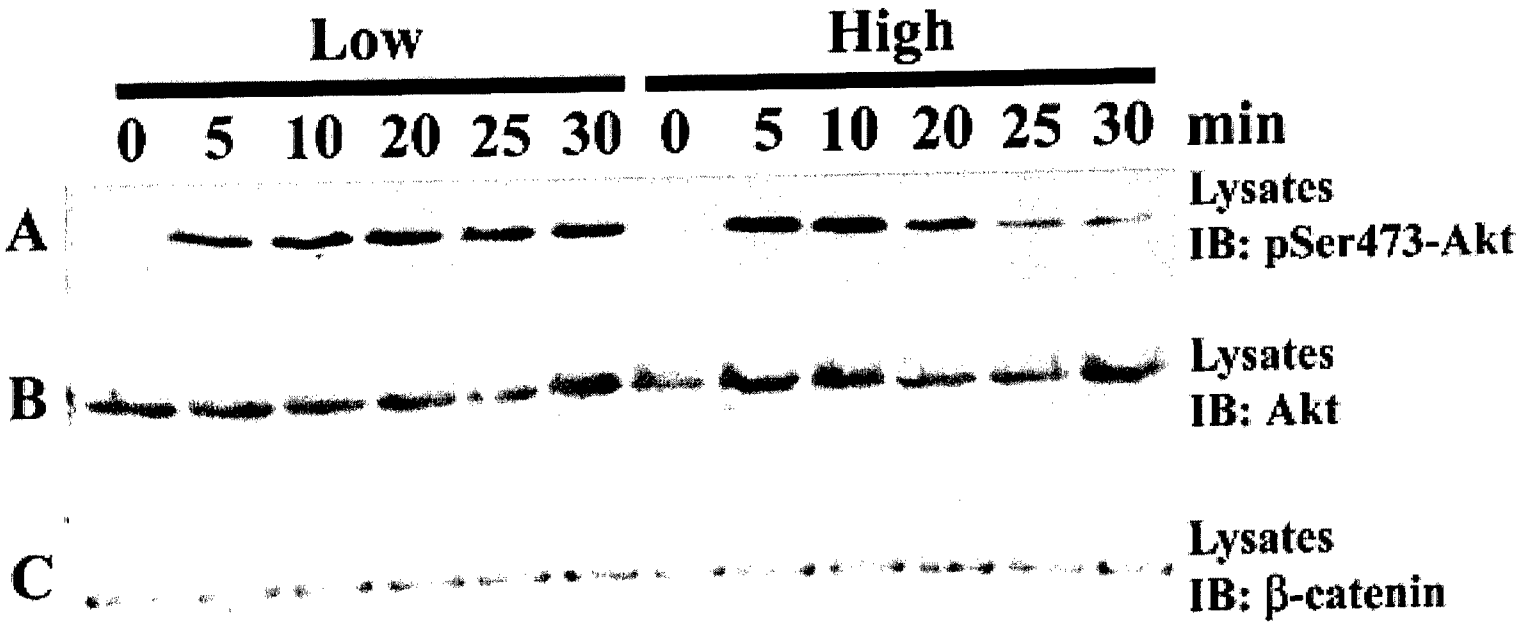

Low
High
0 5 10 20 25 30 0 5 10 20 25 30 min
A
Lysates
IB: PY-EGFR
B
Lysates
IB: EGFR

fig. 2


Low
High
0 5 10 20 25 30 0 5 10 20 25 30 min
A
Lysates
IB: pSer473-Akt
B
Lysates
IB: Akt
C
Lysates
IB: β-catenin

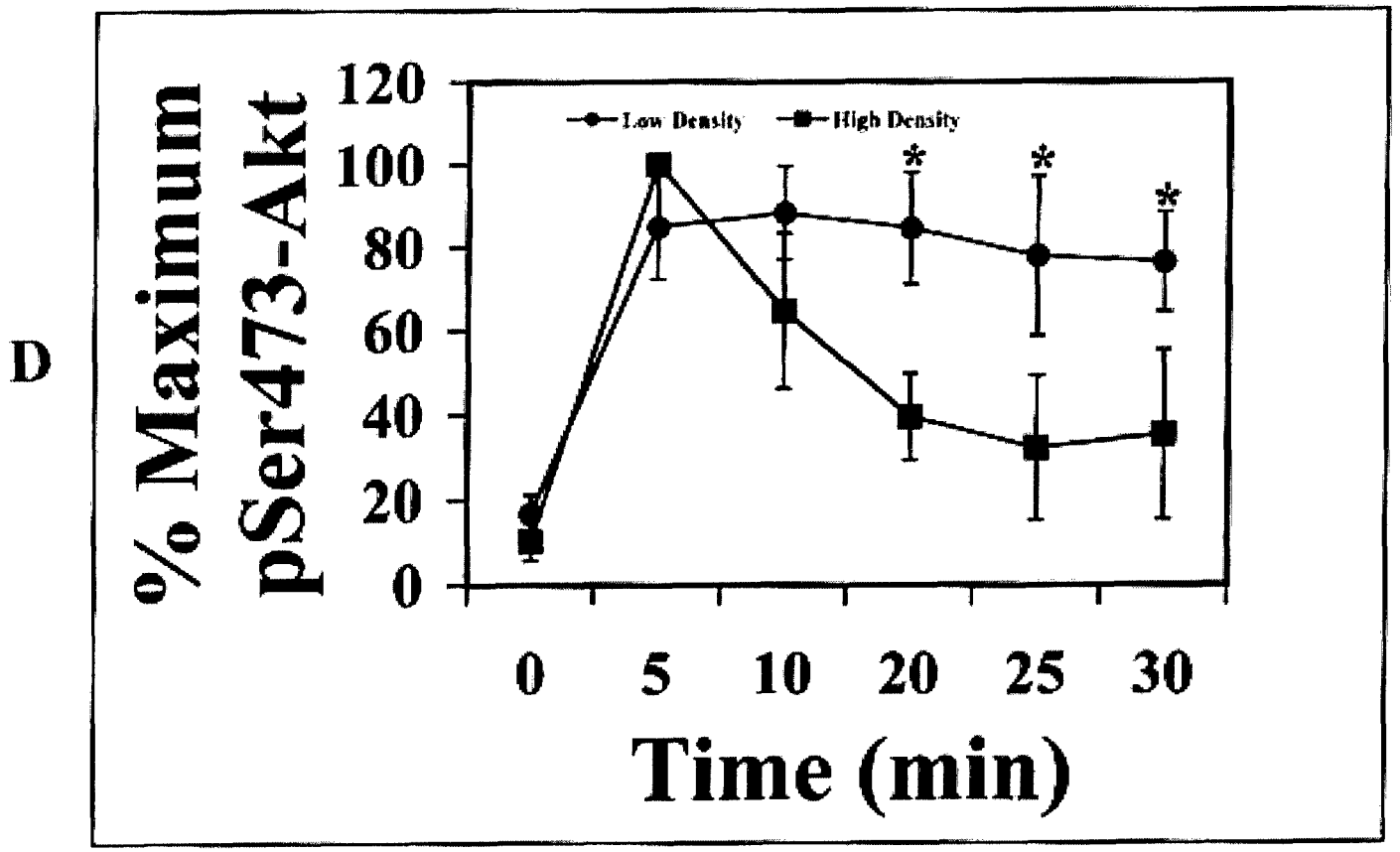

D
% Maximum pSer473-Akt
120
100
80
60
40
20
0
Low Density
High Density
*
*
*
0 5 10 20 25 30
Time (min)

fig. 3

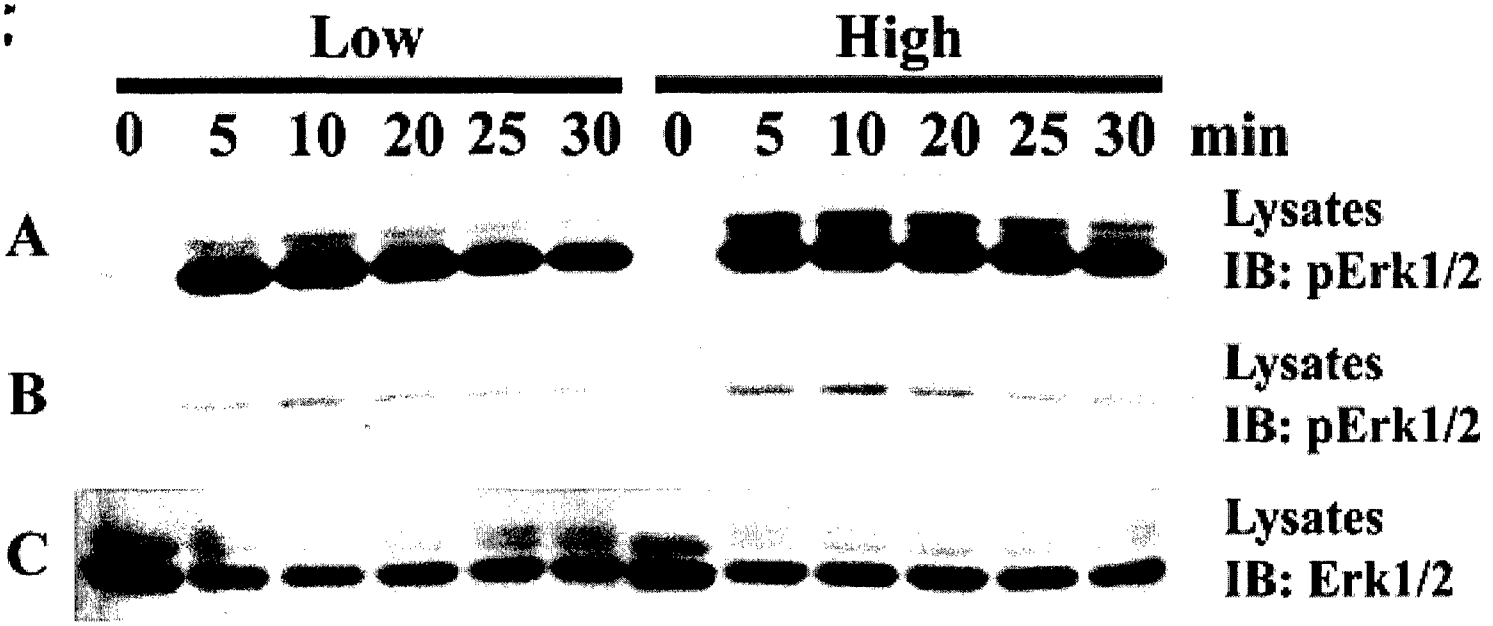

Low
High
0 5 10 20 25 30 0 5 10 20 25 30 min
A
Lysates
IB: pErk1/2
B
Lysates
IB: pErk1/2
C
Lysates
IB: Erk1/2

fig. 4

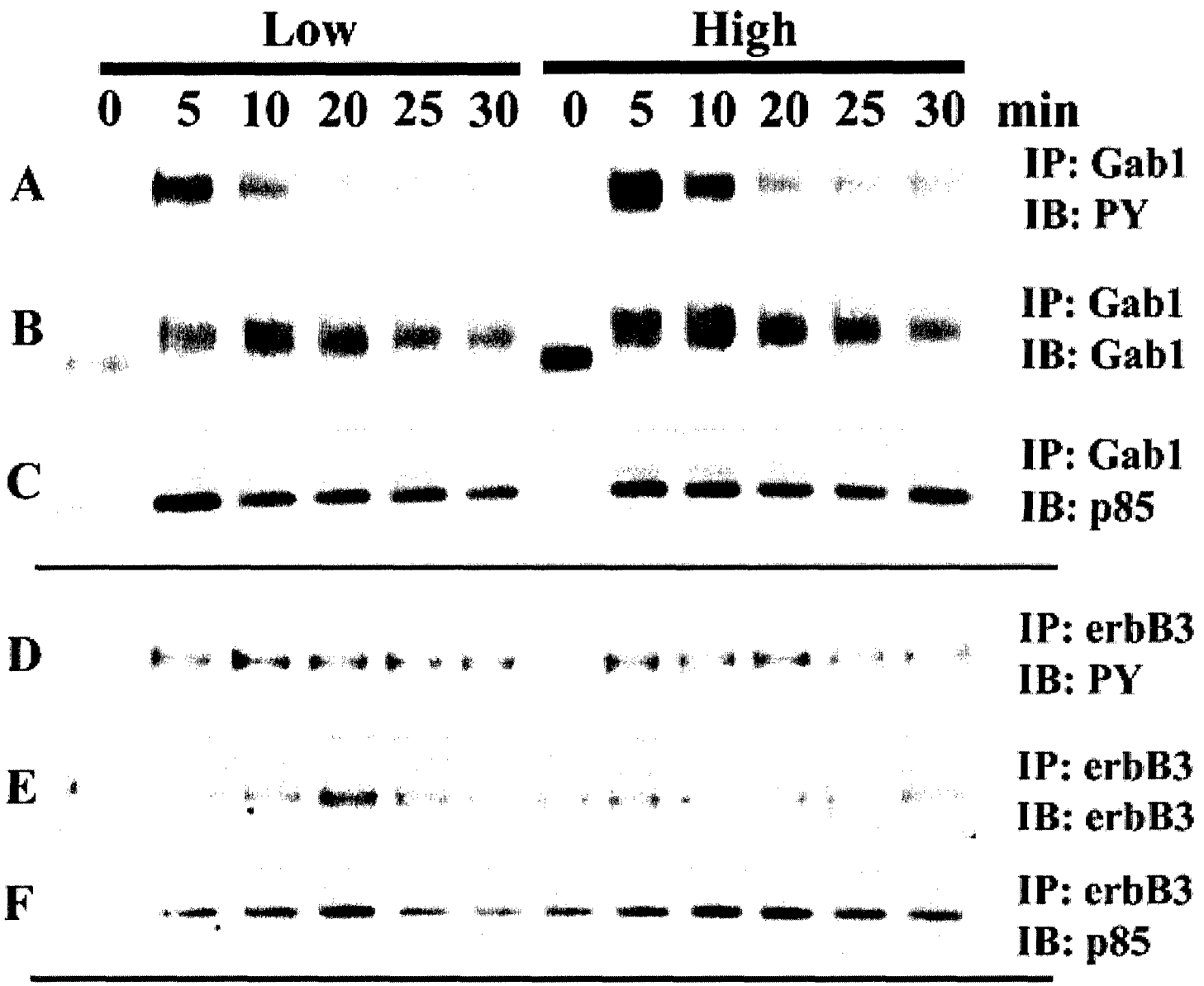

Low
High
0 5 10 20 25 30 0 5 10 20 25 30 min
A
IP: Gab1
IB: PY
B
IP: Gab1
IB: Gab1
C
IP: Gab1
IB: p85
D
IP: erbB3
IB: PY
E
IP: erbB3
IB: erbB3
F
IP: erbB3
IB: p85

fig.5

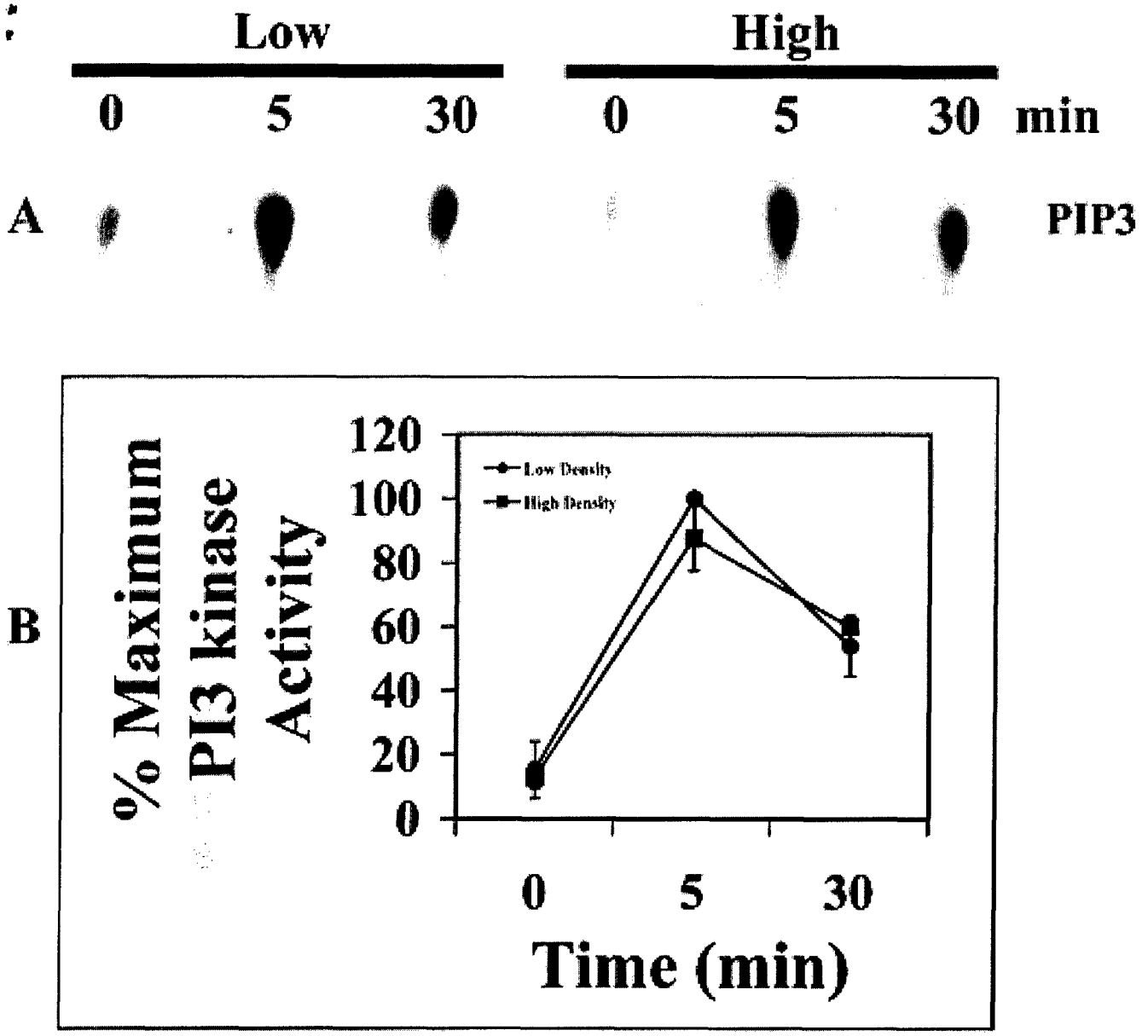

Low
High
0
5
30
0
5
30
min
A
PIP3
B
% Maximum PI3 kinase Activity
120
100
80
60
40
20
0
Low Density
High Density
0
5
30
Time (min)

fig. 6

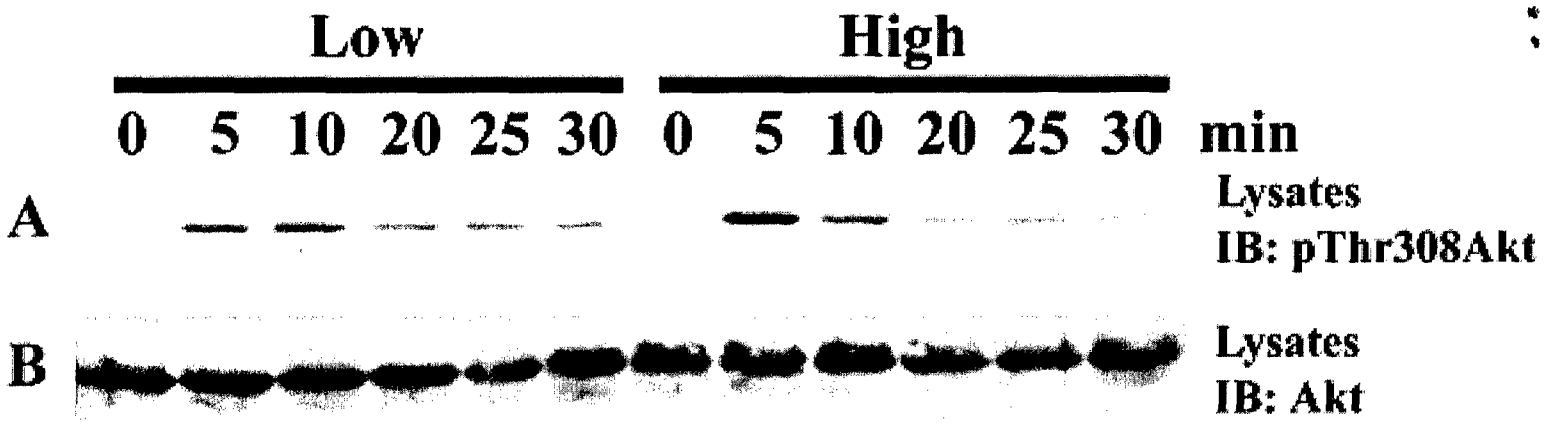

Low
High
0 5 10 20 25 30 0 5 10 20 25 30 min
A
Lysates
IB: pThr308Akt
B
Lysates
IB: Akt

fig. 7

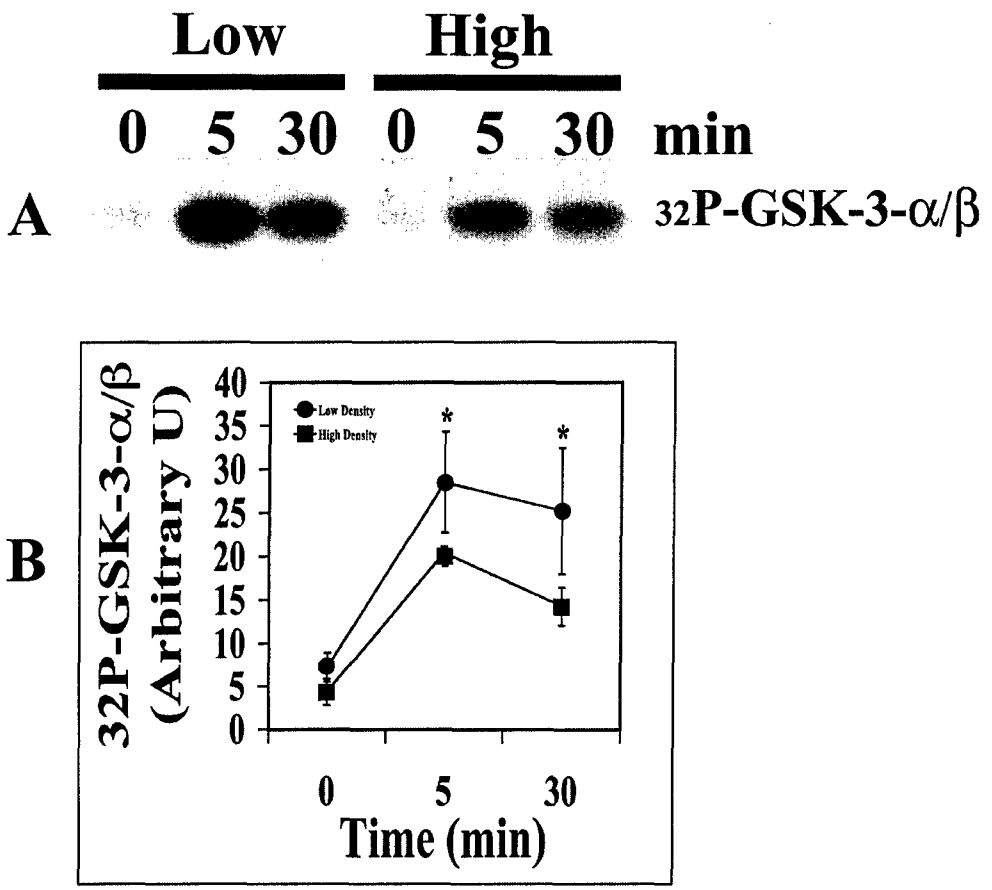

Low
High
0 5 30 0 5 30 min
A
32P-GSK-3-α/β
B
32P-GSK-3-α/β (Arbitrary U)
40
35
30
25
20
15
10
5
0
Low Density
High Density
*
*
0 5 30
Time (min)

fig. 8

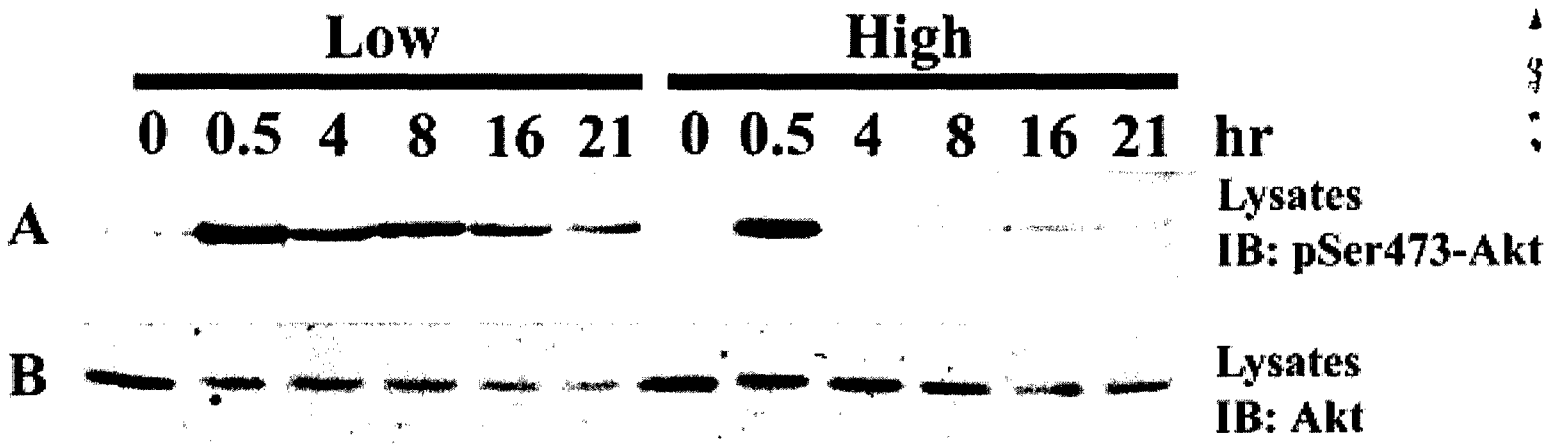

Low
High
0 0.5 4 8 16 21 0 0.5 4 8 16 21 hr
A
Lysates
IB: pSer473-Akt
B
Lysates
IB: Akt

fig. 9

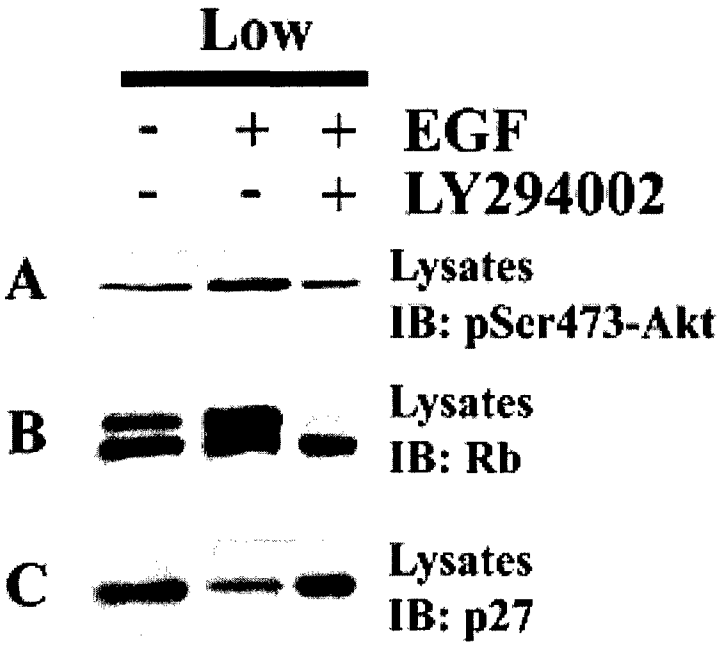

Low
- + + EGF
- - + LY294002
A
Lysates
IB: pSer473-Akt
B
Lysates
IB: Rb
C
Lysates
IB: p27

fig. 10

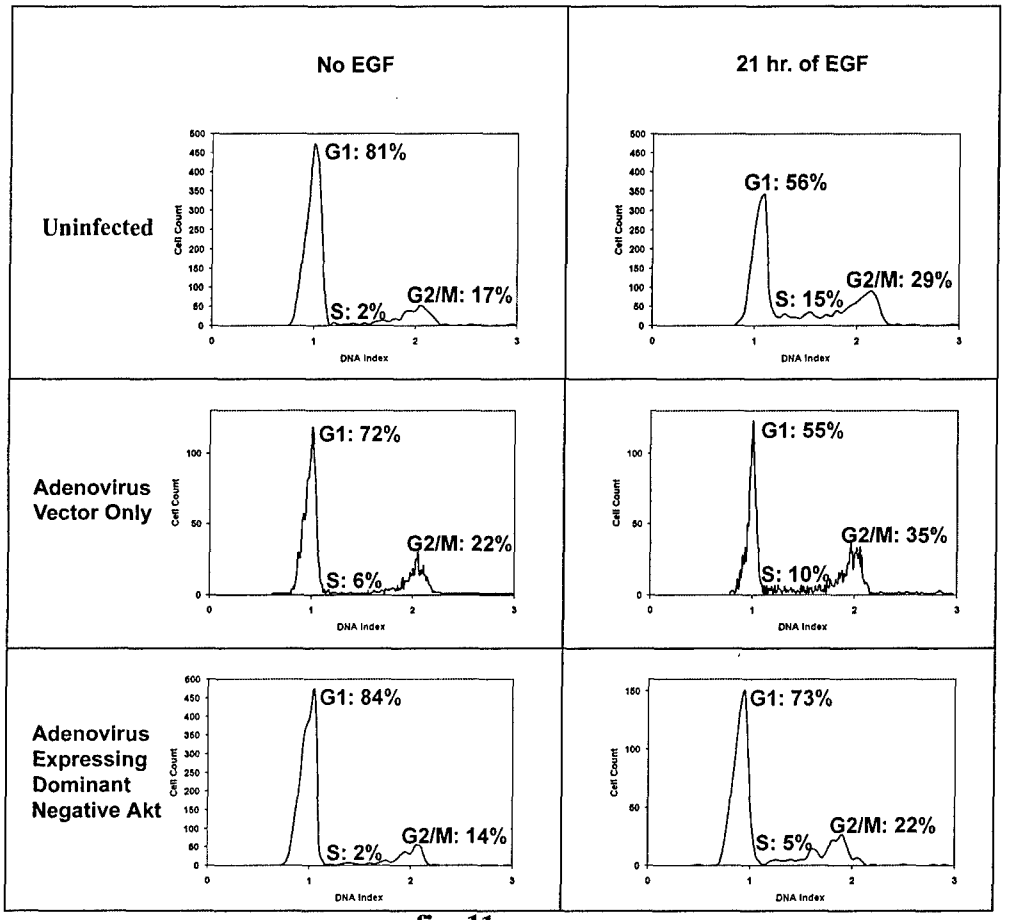

No EGF
21 hr. of EGF
Uninfected
G1: 81%
S: 2%
G2/M: 17%
G1: 56%
S: 15%
G2/M: 29%
Adenovirus Vector Only
G1: 72%
S: 6%
G2/M: 22%
G1: 55%
S: 10%
G2/M: 35%
Adenovirus Expressing Dominant Negative Akt
G1: 84%
S: 2%
G2/M: 14%
G1: 73%
S: 5%
G2/M: 22%
Cell Count
DNA Index

fig. 11